

Date of Deposit December 8, 1998

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Signature: Kimberly W. Zuehlke

Typed Name of Person Mailing Paper or Fee: Kimberly W. Zuehlke

12/08/98

<b>UTILITY PATENT APPLICATION TRANSMITTAL</b>  (Only for new nonprovisional applications under 37 CFR 1.53(b))	Attorney Docket No.	06510/060DIV	Total Pages	84
	First Named Inventor or Application Identifier			
	Shaun R. Couglin; Hiroaki Ishihara; Andrew Connolly			
	Express Mail Label No.	EL 105 872 719 US		

12/08/98

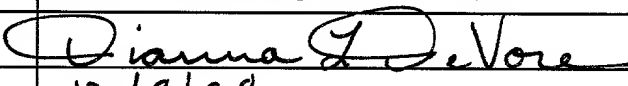
12/08/98

<b>APPLICATION ELEMENTS</b>  See MPEP chapter 600 concerning utility patent application contents	Assistant Commissioner for Patents Address to: Box Patent Application Washington, D.C. 20231
1. <input checked="" type="checkbox"/> Fee Transmittal Form (Submit an original, and a duplicate for fee processing) 2. <input checked="" type="checkbox"/> Specification Total Pages <u>43</u> (preferred arrangement set forth below) - Descriptive title of the invention - Cross Reference to Related Applications - Statement Regarding Fed sponsored R & D - Reference to Microfiche Appendix  - Background of the Invention  - Brief Summary of the Invention - Brief Description of the Drawings (if filed) - Detailed Description - Claim(s) - Abstract of the Disclosure 3. <input checked="" type="checkbox"/> Drawing(s) (35 USC 113) Total Sheets <u>16</u> 4. <input checked="" type="checkbox"/> Oath or Declaration Total Sheets <u>2</u> a. <input type="checkbox"/> Newly executed (original or copy) b. <input checked="" type="checkbox"/> Copy from a prior application (37 CFR 1.63(d) (for continuation/divisional with Box 17 completed) [Note Box 5 below] i. <input type="checkbox"/> <u>DELETION OF INVENTOR(S)</u> Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b) c. <input type="checkbox"/> Unsigned 5. <input checked="" type="checkbox"/> Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.	6. <input type="checkbox"/> Microfiche Computer Program (Appendix) 7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary) a. <input type="checkbox"/> Computer Readable Copy b. <input type="checkbox"/> Paper Copy (identical to computer copy) c. <input type="checkbox"/> Statement verifying identity of above copies  <b>ACCOMPANYING APPLICATION PARTS</b> 8. <input type="checkbox"/> Assignment Papers (cover sheet & document(s)) 9. <input type="checkbox"/> 37 CFR 3.73(b) Statement <input type="checkbox"/> Power of (when there is an assignee) Attorney 10. <input type="checkbox"/> English Translation Document (if applicable) 11. <input type="checkbox"/> Information Disclosure <input type="checkbox"/> Copies of IDS Statement (IDS)/PTO-1449 Citations 12. <input checked="" type="checkbox"/> Preliminary Amendment 13. <input checked="" type="checkbox"/> Return Receipt Postcard (MPEP 503) (Should be specifically itemized) 14. <input type="checkbox"/> Small Entity <input type="checkbox"/> Statement filed in prior application Statement(s) Status still proper and desired 15. <input type="checkbox"/> Certified Copy of Priority Document(s) (if foreign priority is claimed) 16. <input checked="" type="checkbox"/> Other: Submission of Formal Drawings; 16 Sheets of Formal Drawings
17a. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information: <input type="checkbox"/> Continuation <input checked="" type="checkbox"/> Divisional <input type="checkbox"/> Continuation-in-part (CIP) of prior application No. <u>08/742,440</u>	
17b. If a CONVERSION from a PROVISIONAL APPLICATION, supply the requisite information: Conversion of prior provisional application No. 60/_____, filed .	

**UTILITY PATENT APPLICATION TRANSMITTAL**

(Only for new non-provisional applications under 37 CFR 1.53(b))

18. CORRESPONDENCE ADDRESS					
____ Customer Number or Bar code label  (Insert Customer No. or attach bar code label here)		or <u>X</u> Correspondence address below			
NAME	BOZICEVIC & REED LLP				
ADDRESS	285 Hamilton Avenue, Suite 200				
CITY	Palo Alto	STATE	CA	ZIP CODE	94301
COUNTRY	U.S.A.	TELEPHONE	(650) 327-3400	FAX	(650) 327-3231

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT	
NAME	Dianna L. DeVore, Registration No. 42,484
SIGNATURE	
DATE	12/8/98

BOZICEVIC & REED LLP  
 285 Hamilton Avenue, Suite 200  
 Palo Alto, CA 94301  
 Telephone: (650) 327-3400  
 Facsimile: (650) 327-3231

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Signature: Kimberly W. Zuehlke

Typed Name of Person Mailing Paper or Fee: Kimberly W. Zuehlke

<h2 style="margin: 0;">FEE TRANSMITTAL</h2> <p style="margin: 10px 0;"><i>Note: Effective October 1, 1997. Patent fees are subject to annual revision.</i></p>	<b>Complete if Known</b>	
	Application Number	To Be Assigned
	Filing Date	December 8, 1998
	First Named Inventor	Shaun R. Coughlin et al.
	Group Art Unit	Not Assigned Yet
	Examiner Name	Not Assigned Yet
TOTAL AMOUNT OF PAYMENT	(\$916.00)	
	Attorney Docket Number	06510/060DIV

<p><b>METHOD OF PAYMENT (check one)</b></p> <p>1. <input checked="" type="checkbox"/> The commissioner is hereby authorized to charge any additional fees and credit any overpayments to:          Deposit Account No.: 18-0580.          Deposit Account Name: Bozicevic &amp; Reed LLP</p> <p>___ Charge Any Additional ___ Charge the Issue Fee set in          Fee Required Under 37 CFR 1.18 at the mailing          37 CFR 1.16 and 1.17 of the Notice of Allowance</p> <p>2. <input checked="" type="checkbox"/> Payment Enclosed:  <input checked="" type="checkbox"/> Check ___ Money Order ___ Other</p> <p style="text-align: center;"><b>FEE CALCULATION</b></p> <p><b>1. FILING FEE</b></p> <table style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>Large Fee Code</th> <th>Entity Fee (\$)</th> <th>Small Fee Code</th> <th>Entity Fee (\$)</th> <th>Fee Description</th> <th>Fee Due</th> </tr> </thead> <tbody> <tr> <td>101</td> <td>760</td> <td>201</td> <td>380</td> <td>Utility filing fee</td> <td>\$760.00</td> </tr> <tr> <td>102</td> <td>310</td> <td>206</td> <td>155</td> <td>Design filing fee</td> <td></td> </tr> <tr> <td>104</td> <td>480</td> <td>207</td> <td>240</td> <td>Plant filing fee</td> <td></td> </tr> <tr> <td>109</td> <td>760</td> <td>208</td> <td>380</td> <td>Reissue filing fee</td> <td></td> </tr> <tr> <td>110</td> <td>150</td> <td>214</td> <td>75</td> <td>Provisional filing fee</td> <td></td> </tr> <tr> <td colspan="5" style="text-align: right;"><b>SUBTOTAL (1)</b></td> <td><b>760.00</b></td> </tr> </tbody> </table> <p><b>2. CLAIMS</b></p> <table style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th colspan="2"></th> <th colspan="2">Fee from</th> <th></th> <th>Fee Due</th> </tr> <tr> <th colspan="2"></th> <th>Extra</th> <th>below</th> <th></th> <th></th> </tr> </thead> <tbody> <tr> <td>Total Claims</td> <td>7 - 20 =</td> <td>0</td> <td>X</td> <td>18</td> <td>0.00</td> </tr> <tr> <td>Independent Claims</td> <td>5 - 3 =</td> <td>2</td> <td>X</td> <td>78</td> <td>156.00</td> </tr> <tr> <td>Multiple Dependent Claims</td> <td></td> <td>0</td> <td>X</td> <td>260</td> <td>0.00</td> </tr> </tbody> </table> <table style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>Large Fee Code</th> <th>Entity Fee (\$)</th> <th>Small Fee Code</th> <th>Entity Fee (\$)</th> <th>Fee Description</th> <th>Fee Due</th> </tr> </thead> <tbody> <tr> <td>103</td> <td>18</td> <td>203</td> <td>9</td> <td>Claims in excess of 20</td> <td></td> </tr> <tr> <td>102</td> <td>78</td> <td>202</td> <td>39</td> <td>Independent Claims in excess of 3</td> <td></td> </tr> <tr> <td>104</td> <td>260</td> <td>204</td> <td>130</td> <td>Multiple Dependent Claim</td> <td></td> </tr> <tr> <td>109</td> <td>78</td> <td>209</td> <td>39</td> <td>Reissue independent claims over original patent</td> <td></td> </tr> <tr> <td>110</td> <td>18</td> <td>210</td> <td>9</td> <td>Reissue claims in excess of and over original patent</td> <td></td> </tr> <tr> <td colspan="5" style="text-align: right;"><b>SUBTOTAL (2)</b></td> <td><b>\$156.00</b></td> </tr> </tbody> </table>	Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Due	101	760	201	380	Utility filing fee	\$760.00	102	310	206	155	Design filing fee		104	480	207	240	Plant filing fee		109	760	208	380	Reissue filing fee		110	150	214	75	Provisional filing fee		<b>SUBTOTAL (1)</b>					<b>760.00</b>			Fee from			Fee Due			Extra	below			Total Claims	7 - 20 =	0	X	18	0.00	Independent Claims	5 - 3 =	2	X	78	156.00	Multiple Dependent Claims		0	X	260	0.00	Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Due	103	18	203	9	Claims in excess of 20		102	78	202	39	Independent Claims in excess of 3		104	260	204	130	Multiple Dependent Claim		109	78	209	39	Reissue independent claims over original patent		110	18	210	9	Reissue claims in excess of and over original patent		<b>SUBTOTAL (2)</b>					<b>\$156.00</b>	<p><b>3. 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late filing fee or oath		127	50	227	25	Surcharge - late provisional filing fee or cover sheet		139	130	139	130	Non-English specification		147	2,520	147	2,520	For filing a request for reexamination		112	920*	112	920*	Requesting publication of SIR prior to Examiner action		113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action		115	110	215	55	Extension for reply within first month		116	380	216	190	Extension for reply within second month		117	870	217	435	Extension for reply within third month		118	1,360	218	680	Extension for reply within fourth month		128	1,850	228	925	Extension for reply within fifth month		119	300	219	150	Notice of Appeal		120	300	220	150	Filing a brief in support of an appeal		121	260	221	130	Request for oral hearing		138	1,510	138	1,510	Petition to institute a public use proceeding		140	110	240	55	Petition to revive - unavoidable		141	1,210	241	605	Petition to revive - unintentional		142	1,210	242	605	Utility issue fee (or reissue)		143	430	243	215	Design issue fee		144	580	244	290	Plant issue fee		122	130	122	130	Petitions to the Commissioner		123	50	123	50	Petitions related to provisional applications		126	240	126	240	Submissions of Information Disclosure Stmt		581	40	581	40	Recording each patent assignment per property (times number of properties)		146	760	246	380	Filing a submission after final rejection (37 CFR 1.129(a))		149	760	249	380	For each additional invention to be examined (37 CFR 1.129(b))		Other fee (specify)						Other fee (specify)						*Reduced by Basic Filing Fee Paid						<b>SUBTOTAL (3) (\$)</b>					
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Typed or Printed Name	Dianna L. DeVore			Reg. Number	42,484
Signature	<u>Dianna L. DeVore</u>			Date	12/8/98
				Deposit Account	18-0580

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Shaun R. Coughlin et al.

Group Art Unit: Unassigned

Serial No.: Unassigned (Divisional of USSN 08/742,440)

Examiner: Unassigned

Filing Date: December 8, 1998

Title: PROTEASE-ACTIVATED RECEPTOR 3 AND  
USES THEREOF

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

This is a preliminary amendment to the patent application identified above. Prior to examination of the subject application, please enter the following amendments to the specification and claims:

IN THE SPECIFICATION:

On page 1, beneath the title, please insert the following:

--CROSS-REFERENCE

This application is a divisional application of Serial No. 08/742,440, filed October 30, 1996 which is incorporated herein by reference in its entirety and to which application we claim priority under 35 U.S.C. §120.--

IN THE CLAIMS:

Please cancel claims 1-4, 9-12 and 16-20 without prejudice.

REMARKS

Claims 5-8 and 13-15, which correspond to Restriction Groups II and IV, respectively, from the prosecution of parent application 08/742,440, are now pending in this application.

Original claims 1-4, 9-12 and 16-20 have been canceled from the application

During the prosecution of the parent application a restriction requirement was issued which resulted in the cancellation of original claims 5-8 and 13-15. This application has been filed to claim subject matter encompassed by these canceled claims.

In the event any fees are due in connection with the filing of this preliminary amendment or attached application or if petitions are required, applicants petition for any required relief and authorize the Commissioner to charge the cost of such petitions or other fees to our Deposit Account No. 18-0580.

Respectfully submitted,

Date: 12/8/98

By: Dianna L. DeVore  
Dianna L. DeVore  
Registration No. 42,484

BOZICEVIC & REED LLP  
285 Hamilton Avenue, Suite 200  
Palo Alto, CA 94301  
Telephone: (650) 327-3400  
Fax: (650) 327-3231

F:\DOCUMENT\6510 - UCAL\060DIV\Preliminary Amendment wpd (Rev. 6/19/97)

**APPLICATION**  
**FOR**  
**UNITED STATES LETTERS PATENT**


**TITLE:** **PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF**

**APPLICANT:** **SHAUN R. COUGHLIN**  
**HIROAKI ISHIHARA**  
**ANDREW CONNOLLY**

"EXPRESS MAIL" Mailing Label Number EM344048760US

Date of Deposit October 30, 1996

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Burt A. Villalba

## PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF

### Field of the Invention

5           This invention relates to nucleic acids, their encoded protease-activated receptor 3 proteins, and screening assays for agonists and antagonists of the protease activated receptor 3 proteins.

### Background of the Invention

10           Thrombin, a coagulation protease generated at sites of vascular injury, activates platelets, leukocytes, and mesenchymal cells (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068). Activation of platelets by thrombin is thought to be critical for hemostasis and  
15 thrombosis. In animal models, thrombin inhibitors block platelet-dependent thrombosis, which is the cause of most heart attacks and strokes in humans. Available data in humans suggests that thrombosis in arteries can be blocked by inhibitors of platelet function and by  
20 thrombin inhibitors. Thus it is likely that thrombin's actions on platelets contribute to the formation of clots that cause heart attack and stroke. Thrombin's other actions on vascular endothelial cells and smooth muscle cells, leukocytes, and fibroblasts may mediate  
25 inflammatory and proliferative responses to injury, as occur in normal wound healing and a variety of diseases (atherosclerosis, restenosis, pulmonary inflammation (ARDS), glomerulosclerosis, etc.). A thorough understanding of how thrombin activates cells is an  
30 important goal.

A receptor that mediates thrombin signaling has been previously identified (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068; USPN 5,256,766). This receptor revealed a novel proteolytic mechanism of activation and is referred to as PAR1 (protease-activated receptor 1). PAR1 is activated by the binding of thrombin to and cleavage of PAR1's amino terminal exodomain at a specific site. Receptor cleavage unmask a new amino terminus, which then functions as a tethered peptide ligand by binding intramolecularly to the body of the receptor to effect transmembrane signaling (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068). Synthetic peptides that mimic this tethered ligand domain function as PAR1 agonists and activate it independent of thrombin and receptor cleavage (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068).

To identify which of thrombin's known cellular actions are mediated by PAR1, a PAR1 knockout mouse was recently generated (Connolly, A. et al. (1996) Nature 381:516-519). Analysis of mice in which both alleles of the PAR1 gene were disrupted provided definitive evidence for a second platelet thrombin receptor and for tissue specific roles of distinct thrombin receptors. Specifically, in mice, PAR1 is not important for platelet responses but is critical for fibroblast responses.

A second protease-activated receptor (PAR2) was cloned during a search for relatives of the Substance K receptor (Nystedt, S., et al. (1994) PNAS USA, 91:9208-9212). The physiological activator of PAR2 remains unknown; it is not activated by thrombin.

30

#### SUMMARY OF THE INVENTION

The protease-activated receptor (PAR3) disclosed herein is useful in assaying libraries of compounds for their activity as thrombin agonists and antagonists. DNA



encoding PAR3 is placed in a functional expression vector, expressed in a cell line, and used to assay compounds for activity as an agonist or antagonist of thrombin's affect on PAR3.

5           The invention features substantially pure DNA (cDNA or genomic DNA) encoding a protease-activated receptor 3 (PAR3) from vertebrate tissues (SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5) and degenerate sequences thereof; substantially pure protease-activated  
10 receptor 3 polypeptides encoded thereby; as well as amino acid sequences substantially identical to the amino acid sequences SEQ ID NO:3 and SEQ ID NO:6 from mouse and human, respectively. The invention further comprises fragments of the PAR3 receptor which are activated by  
15 thrombin. Such fragments may have the same amino acid sequence as SEQ ID NOs:3 and 6 or be at least 80% identical to the amino acid sequences SEQ ID NO:3 and SEQ ID NO:6.

          In various embodiments, the DNA, receptor or  
20 receptor fragment is derived from a vertebrate animal, preferably, human or mouse. However, the gene can be chemically synthesized.

          An object of the invention is to provide a nucleotide sequence encoding a novel receptor.

25           Another object is to provide a cell line genetically engineered to express the nucleotide sequence.

          Another object is to provide a method whereby a compound or library of compounds can be assayed as  
30 thrombin agonists or antagonists for their ability to activate or block the receptor expressed by the nucleotide sequence.

          An advantage of the present invention is that a novel thrombin receptor PAR3 is disclosed making it  
35 possible to identify novel thrombin agonists and

antagonists which may not be identifiable via PAR1 or PAR2 receptors.

A feature of the invention is that it makes it possible to obtain additional information regarding  
5 thrombin activation and the sequence of biochemical events initiated by such.

These and other objects, advantages and features of the present invention will become apparent to those skilled in the art upon reading the disclosure.

10

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is the complete nucleotide and amino acid sequences (SEQ ID NO:1 and SEQ ID NO:3, respectively) of the mouse protease-activated receptor 3 gene coding region cDNA. The deduced amino acid sequence of the  
15 receptor is provided below the nucleotide sequence and contains 369 amino acids. The deduced amino acid sequence begins at nucleotides 51-53 (ATG = Met) and ends at nucleotides 1158-1160 (TAG = stop).

Fig. 2 is the genomic sequence (containing exon 2)  
20 of the mouse protease-activated receptor 3 (SEQ ID NO:2).

Fig. 3 is the nucleotide and deduced amino acid sequences (SEQ ID NO:4 and SEQ ID NO:6, respectively) of the human protease-activated gene coding region cDNA. The deduced amino acid sequence is provided below the  
25 nucleotide sequence and contains 374 amino acids. The coding region of the cDNA sequence begins at nucleotides 58-60 (ATG = Met) and ends at nucleotides 1180-1182 (TAG = stop).

Fig. 4 is the genomic sequence (containing exon 2)  
30 of the human protease-activated receptor 3 (SEQ ID NO:5).

Fig. 5A shows the alignment of the deduced amino acid sequences (SEQ ID NO:3, 6, 7, 8, 9) of the mouse PAR3, human PAR3, human PAR1, and human PAR2. To

indicate homology, gaps (represented by blank spaces) have been introduced into the five sequences. Transmembrane domains are overlined (TM1-7). Fig. 5B shows the alignment of the hirudin-like portion of human  
5 PAR1, PAR2, and PAR3 amino acid sequences.

Fig. 6 is a bar graph showing cell surface binding of M1 monoclonal antibody to M1 epitope on Cos 7 cells expressing hPAR3 or hPAR3 T39P in the presence and absence of  $\alpha$ -thrombin.

10 Fig. 7 is a bar graph of hPAR3 signaling in Cos 7 cells in the presence and absence of G $\alpha$ 16 and the presence and absence of  $\alpha$ -thrombin. Signaling is measured by phosphoinositide hydrolysis.

Fig. 8 is a graph of phosphoinositide hydrolysis  
15 in response to PAR3 signaling as a function of increasing  $\alpha$ -thrombin concentration, and in the presence and absence of G $\alpha$ 16 protein.

Fig. 9 is a graph of phosphoinositide hydrolysis in response to PAR3 signaling as a function of increasing  
20  $\gamma$ -thrombin concentration, and in the presence and absence of G $\alpha$ 16 protein.

Fig. 10 is a graph comparing the specificity of PAR1 and PAR3 for thrombin.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

25 Before the present protease-activated receptor assays and methods of using such are described, it is to be understood that this invention is not limited to the particular DNA sequences, materials, methods, or processes described as such may, of course, vary. It is  
30 also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope

of the present invention will be limited only by the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms  
5 "a", "and," and "the" include plural referents unless the contexts clearly dictates otherwise. Thus, for example, reference to "a DNA sequence" includes mixtures and large numbers of such sequences, reference to "an assay" includes assays of the same general type, and reference  
10 to "the method" includes one or more methods or steps of the type described herein.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be  
15 construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly  
20 understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials  
25 are described herein. All publications cited herein are incorporated herein by reference for the purpose of disclosing and describing specific aspects of the invention for which the publication is cited in connection with.

30

#### DEFINITIONS

By "protease-activated receptor 3", "PAR3", "PAR3 receptor" and the like, is meant all or part of a vertebrate cell surface protein which is specifically

activated by thrombin or a thrombin agonist thereby activating PAR3-mediated signalling events (e.g., phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, platelet aggregation). The polypeptide is characterized as having  
5 the ligand activating properties (including the agonist activating and antagonist inhibiting properties) and tissue distribution described herein. Specifically, PAR3 receptors are expressed by the DNA sequences of SEQ ID NOs:2, 4, and 5.

10 By a "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation).

By "substantially pure" is meant that the protease-activated receptor 3 polypeptide provided by the  
15 invention is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, PAR3  
20 polypeptide. A substantially pure PAR3 polypeptide may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding a PAR3 polypeptide, or by chemically synthesizing the protein. Purity can be measured by any  
25 appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. The protein is substantially pure if it can be isolated to a band in a gel.

By a "substantially identical" amino acid sequence  
30 is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for leucine, arginine for lysine, etc.) or by one or more non-conservative amino acid  
35 substitutions, deletions, or insertions located at

positions of the amino acid sequence which do not destroy the biological activity of the receptor. Such equivalent receptors can be isolated by extraction from the tissues or cells of any animal which naturally produce such a  
5 receptor or which can be induced to do so, using the methods described below, or their equivalent; or can be isolated by chemical synthesis; or can be isolated by standard techniques of recombinant DNA technology, e.g., by isolation of cDNA or genomic DNA encoding such a  
10 receptor. Substantially identical receptors have the same biological function, e.g. are activated by the same compound.

By "derived from" is meant encoded by the genome of that organism and present on the surface of a subset  
15 of that organism's cells.

By "isolated DNA" is meant DNA that is not in its native environment in terms of not being immediately contiguous with (i.e., covalently linked to) the complete coding sequences with which it is immediately contiguous  
20 (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the DNA of the invention is derived. The term therefore includes, for example, recombinant DNA which is incorporated into a vector; into an autonomously  
25 replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes any  
30 recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

"Isolated DNA" can mean the DNA is in vectors which are preferably capable of directing expression of the protein encoded by the DNA in a vector-containing  
35 cell and further includes cells containing such vectors

(preferably eukaryotic cells, e.g., CHO cells (ATCC; Cat. No. CCL 61 or COS-7 cells (ATCC; Cat. No. CRL 1651; and the *Xenopus* oocytes of the type described in the above cited reference Vu, T.-K.H. et al. (1991) Cell 64:1057-  
5 1068). Preferably, such cells are stably transfected with such isolated DNA.

By "transformed cell" and "transfected cell", "genetically engineered cell", and the like, is meant a cell into which (or into an ancestor of which) has been  
10 introduced, by means of genetic engineering, a DNA molecule encoding a PAR3 (or DNA encoding a biologically active fragment or analog, thereof). Such a DNA molecule is "positioned for expression" meaning that the DNA molecule is positioned adjacent to a DNA sequence which  
15 directs transcription and translation of the sequence (i.e., facilitates the production of the PAR3 protein, or fragment or analog, thereof).

By "specifically activates", as used herein, is meant an agent, such as thrombin, a thrombin analog, a  
20 PAR3 agonist or other chemical agent including polypeptides such as an antibody, which activates protease-activated receptor 3, receptor polypeptide or a fragment or analog thereof to initiate PAR3-mediated biological events as described herein, but which does not  
25 substantially bind other molecules in a sample, e.g., a biological sample, which naturally includes a protease-activated receptor 3 polypeptide.

By "specifically inhibits", as used herein, is meant an agent, such as a thrombin analog, a PAR3  
30 antagonist or other chemical agent including polypeptides such as an antibody, which inhibits activation of protease-activated receptor 3, receptor polypeptide or a fragment or analog thereof, such as by inhibiting thrombin or by blocking activation of PAR3 by thrombin or  
35 other PAR3 activator. Preferably, the agent activates or

inhibits the biological activity *in vivo* or *in vitro* of the protein to which it binds.

By "biological activity" is meant the ability of the protease-activated receptor 3 to bind thrombin or a PAR3 agonist and signal the appropriate cascade of biological events (e.g., phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, and platelet aggregation, and the like, as described herein.

By "substantial increase" is meant an increase in activity or other measurable phenotypic characteristic that is at least approximately a 2-fold increase over control level (where control assays are performed in the absence of activator), preferably at least approximately a 5-fold increase, more preferably at least approximately a 10-fold increase in activity over a control assay.

By "substantial decrease" or "substantial reduction" is meant a decrease or reduction in activity or other measurable phenotypic characteristic that is approximately 80% or the control level, preferably reduced to approximately 50% of the control level, or more preferably reduced to approximately 10% or less of the control level.

The terms "screening method" and "assay method" are used to describe a method of screening a candidate compound for its ability to act as an agonist of a PAR3 ligand. The method involves: a) contacting a candidate agonist compound with a recombinant protease-activated receptor 3 (or PAR3 agonist-binding fragment or analog); b) measuring activation of the receptor, the receptor polypeptide or the receptor fragment or analog; and c) identifying agonist compounds as those which interact with the recombinant receptor and trigger PAR3 activation. Interaction may be cleavage of the receptor to unmask an intramolecular receptor activating peptide or by mimicking the intramolecular receptor-activating



peptide. A tethered ligand may be more difficult to block than a free agonist. Thus, blocking thrombin is the acid test for an agonist which will block responses by other thrombin substrates.

5 By an "agonist" is meant a molecule which mimics a particular activity, in this case, interacting with a PAR3 ligand in a manner which activates thereby triggering the biological events which normally result from the interaction (e.g., phosphoinositide hydrolysis, 10  $\text{Ca}^{2+}$  efflux, and platelet aggregation). Preferably, an agonist initiates a substantial increase in receptor activity relative to control assays in the absence of activator or candidate agonist. An agonist may possess the same, less, or greater activity than a naturally- 15 occurring PAR3 ligand.

The terms "antagonist assay", "antagonist screening" and the like, refer to a method of screening a candidate compound for its ability to antagonize interaction between a naturally-occurring activating 20 ligand or an agonist and the PAR3. The method involves: a) contacting a candidate antagonist compound with a first compound which includes a recombinant PAR3 (or agonist-binding fragment or analog) on the one hand and with a second compound which includes thrombin or a PAR3 25 agonist on the other hand; b) determining whether the first and second compounds interact or are prevented from interaction by the candidate compound; and c) identifying antagonistic compounds as those which interfere with the interaction of the first compound (PAR3 receptor) to the 30 second compound (PAR3 agonist) and which thereby substantially reduce thrombin or PAR3 agonist-activated biological events (e.g., phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, and platelet aggregation).

By an "antagonist" is meant a molecule which 35 blocks activation of a PAR3 receptor. This can be done

by inhibiting a particular activity such as the ability of thrombin, for example, to interact with a protease-activated receptor 3 thereby triggering the biological events resulting from such an interaction (e.g.,  
5 phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, and platelet secretion, or platelet aggregation). An antagonist may bind to and thereby block activation of a PAR3 receptor.

The terms "treatment", "treating", "treat" and the like are used herein to generally mean obtaining a  
10 desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the  
15 disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particular a human, and includes:

- (a) preventing the disease or symptom from occurring in a subject which may be predisposed to the  
20 disease or symptom but has not yet been diagnosed as having it;
- (b) inhibiting the disease symptom, i.e., arresting its development; or
- (c) relieving the disease symptom, i.e., causing  
25 regression of the disease.

#### PREFERRED EMBODIMENTS

In preferred embodiments of both screening methods, the recombinant PAR3 is stably expressed by a vertebrate cell which normally presents substantially no  
30 PAR3 on its surface (i.e., a cell which does not exhibit any significant thrombin-mediated phosphoinositide hydrolysis or  $\text{Ca}^{2+}$  efflux in the presence of a PAR activator); the vertebrate cell is a mammalian cell, is a

Rat 1 cell, or a COS 7 cell; and the candidate antagonist or candidate agonist is a thrombin analog, PAR3 peptide fragment or analog or other chemical agent including a polypeptide such as an antibody.

5           The receptor proteins of the invention are likely involved in the activation of vertebrate platelet, leukocyte, and mesenchymal cells in response to wounding, as well as mediating signalling in embryonic development. Such proteins and in particular PAR3 antagonists are  
10 useful therapeutics for the treatment of such conditions as thrombosis, atherosclerosis, restenosis, and inflammation associated with normal wound healing and a variety of diseases including atherosclerosis, restenosis, pulmonary inflammation (ARDS) and  
15 glomerulosclerosis. Preferred therapeutics include 1) agonists, e.g., thrombin analogs, PAR3 peptide fragments or analogs thereof, or other compounds which mimic the action of thrombin upon interaction with the protease-activated receptor 3 or mimic the action of an  
20 intramolecular receptor activating peptide; and 2) antagonists, e.g., thrombin analogs, antibodies, or other compounds, which block thrombin or protease-activated receptor 3 function by interfering with the thrombin:receptor interaction or by interfering with the  
25 receptor intramolecular activating peptide. The dosage would be expected to be comparable with current anti-inflammatory drugs and should be adjusted based on the age, sex, weight and condition of the patient beginning with small doses and increasing gradually based on  
30 responsiveness and toxicity.

Because the receptor component may now be produced by recombinant techniques and because candidate agonists and antagonists may be screened using transformed, cultured cells, the instant invention provides a simple  
35 and rapid approach to the identification of useful

therapeutics. Isolation of the PAR3 gene (as cDNA or genomic DNA) allows its expression in a cell type which does not normally bear PAR3 on its surface, providing a system for assaying a thrombin:receptor interaction and  
5 receptor activation.

#### EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make  
10 receptor proteins and sequences encoding such proteins and carry out the methodology for finding such DNA sequences and proteins, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to insure accuracy with respect to numbers used  
15 (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts or parts by weight, molecular weight is weight average molecular weight; temperature is in degrees centigrade; and pressure is at or near  
20 atmospheric.

There now follows a description of the cloning and characterization of the cDNA, genomic DNA and the receptor protein of the protease-activated receptor 3 from mouse and human. Expression vectors containing and  
25 capable of expressing the PAR3 DNA, as well as transformed cells containing and expressing the DNA of the invention are also described. Also described are possible PAR3 agonists and antagonists as well as screening assays for receptor agonists and receptor  
30 antagonists.

# EXAMPLE 1

## Isolation of the Mouse Protease-Activated Receptor 3

Rat platelets were used as a source of RNA in the search for and cloning of PAR3 because rat platelets are more abundant than mouse platelets and, like mouse platelets, they do not respond to PAR1 agonist peptides (Connolly, A. et al. (1996) *Nature* 381: 516-519; and Connolly, T.M. et al (1994) *Thromb Haemost* 72: 627-33).

Total RNA was prepared from rat platelets using Trizol reagent (Gibco BRL). cDNA was then prepared using random hexamer primers and the Superscript reverse transcriptase system (Gibco, BRL). cDNA was then used as template for PCR amplification using a Robocycler Gradient 96® (Stratagene) and the primers 5'-  
15 GTITACATGCTI(A/C)AC(C/T)TIGCI(A/C/G/T)TIGC(A/C/G/T)GA-3' (SEQ ID NO:10) and 5'-  
GGATAIACIACIGCIA(A/G/T)(A/G)(A/T)AIC(G/T)(A/C/G/T)TC-3' (SEQ ID NO:11) at 5µM in 20µM Tris-HCl (pH 8.4), 50µM KCl, 1.5µM MgCl<sub>2</sub>, 0.2µM dNTP, and 50U/µl Taq polymerase.  
20 Polymerase chain reaction temperature was varied as follows: 94°C for 4 min; 30 cycles of 94°C for 45 sec, 39°C for 60 sec, and 72°C for 90 sec; then 72°C for 7 min. PCR products were subcloned using the TA cloning kit (Invitrogen, San Diego, CA). Rat cDNA clones with  
25 inserts of approximately 200 bp were analyzed by nucleic acid sequencing. One sequence predicted a novel G-protein coupled receptor related to PAR1 and PAR2. This sequence was used to obtain mouse and human cDNA and genomic clones by a combination of PCR and hybridization  
30 techniques (see, for example, Sambrook, J. et al. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York). The nucleotide sequences are shown in Figures 1-4.

The rat PCR product was then used to clone the full length mouse cDNA and genomic DNA clones. The nucleotide sequences and deduced amino acid sequence of the mouse PAR3 are shown in Figs. 1 and 2.

5       The human PAR3 cDNA used for the functional studies presented below was cloned from a Lamda gt 10 intestinal cDNA library (Clonetech). Features of human PAR3's amino acid sequence are shown in Figs. 5A and 5B by alignment of the deduced amino acid sequence of PAR3  
10 with those of PAR1 and PAR2. Predicted transmembrane (TM) domains are overlined and predicted Asn-linked glycosylation sites in PAR3 are underlined in the figure. The amino terminal exodomains are compared in Fig. 5b, including the cleavage site (^), the tethered ligand  
15 domains of PAR1 and PAR2, and the predicted tethered ligand domain of PAR3 (underlined). Also underlined is PAR3's hirudin-like domain (FEEFP). The similar FEEIP and YEPFW sequences in hirudin and PAR1, respectively are known to bind thrombin's fibrinogen-binding exosite.

20       The human PAR3 cDNA contained an open reading frame encoding a 374 amino acid putative G protein-coupled receptor (Fig. 3). BLAST search of the Genbank and EST databases revealed this protein to be novel with 28% and 30% amino acid sequence identity to human PAR1  
25 and PAR2 (Fig. 5a, Table I). Its amino terminal exodomain revealed a possible thrombin cleavage site and a striking hirudin-like sequence (Fig. 5b). Like the carboxyl tail of hirudin itself, PAR1's hirudin-like sequence is known to dock with thrombin's fibrinogen  
30 binding exosite, an interaction important for efficient PAR1 cleavage by thrombin (Vu, T.-K.H. et al. (1991) Nature 353:674-677; Liu, L. et al. (1991) J. Biol. Chem 266:16977-16980; Mathews, I.I. et al. (1994) Biochem 33 3266-79; Ishii, K. (1995) J. Biol. Chem 270:16435-16440,  
35 which references are herein incorporated by reference in

their entirety). These observations strongly suggested that this new receptor was a novel thrombin receptor.

A comparison of PAR deduced amino acid sequences from human, mouse, and *Xenopus* is provided in Table I below. The % identity of the total sequence as well as the % identity of the transmembrane regions are shown.

TABLE I

PAR SEQUENCE	% AMINO ACID IDENTITY	
	TOTAL	TM1-7
hPAR3 vs hPAR1	28	37
hPAR3 vs hPAR2	30	38
hPAR1 vs hPAR2	28	42
hPAR3 vs xPAR1	29	38
hPAR1 vs xPAR1	52	63
hPAR3 vs mPAR3	67	74
hPAR1 vs mPAR1	77	81
hPAR2 vs mPAR2	78	85

h = human      m = mouse      x = *Xenopus laevis*

#### EXAMPLE 2

##### Polypeptide Expression

Polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of a PAR3 encoding cDNA fragment (e.g., the cDNAs described above) in a suitable expression vehicle, and expression of the receptor.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant receptor protein. The precise host cell used is not critical to the invention. The receptor may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae* or mammalian cells, e.g., COS-6M, COS-7, NIH/3T3, or Chinese Hamster Ovary cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockville, MD). The method of transfection and the choice of expression vehicle will depend on the host system selected. Transformation and mammalian cell transfection methods are described, e.g., in Ausubel et al. (Current Protocols in Molecular Biology, John Wiley & Sons, New York, (1989)); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (Pouwels, P.H. et al., (1985), Supp. 1987).

Particularly preferred expression systems are the *Xenopus* oocyte cells of Vu et al. (Vu et al., Cell (1991) *supra*) and insect cells (SF9-baculovirus) transfected with an expression vector containing and expressing a receptor protein or biologically active fragment thereof. DNA encoding the human or mouse PAR3 or an appropriate receptor fragment or analog (as described above) is inserted into the expression vector in an orientation designed to allow expression. Alternatively, the PAR3 (or biologically active receptor fragment or analog) is expressed by a stably-transfected mammalian cell line. Other preferable host cells which may be used in conjunction with the expression vehicle include NIH/3T3 cells (ATCC Accession No. 1658). The expression may be used in a screening method of the invention (described below) or, if desired, the recombinant receptor protein may be isolated as described below.



A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding the receptor (or receptor fragment or analog) is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the PAR3-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300  $\mu$ M methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

One particularly preferred stable expression system is a Rat 1 cell (ATCC) stably transfected with a pcDNAI/NEO (InVitrogen, San Diego, CA) expression vector.

Expression of the recombinant receptor (e.g., produced by any of the expression systems described herein) may be assayed by immunological procedures, such as Western blot or immunoprecipitation analysis of recombinant cell extracts, or by immunofluorescence of

intact recombinant cells (using, e.g., the methods described in Ausubel et al., supra). Recombinant receptor protein is detected using an antibody directed to the receptor. Described below are methods for  
5 producing anti-protease-activated receptor 3 antibodies using, as an immunogen, the intact receptor or a peptide which includes a suitable protease-activate receptor 3 epitope. To detect expression of a PAR3 fragment or analog, the antibody is preferably produced using, as an  
10 immunogen, an epitope included in the fragment or analog.

Once the recombinant PAR3 protein (or fragment or analog, thereof) is expressed, it is isolated, e.g., using immunoaffinity chromatography. In one example, an anti-PAR3 antibody may be attached to a column and used  
15 to isolate intact receptor or receptor fragments or analogs. Lysis and fractionation of receptor-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired,  
20 be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, (1980)).

Receptors of the invention, particularly short  
25 receptor fragments, can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, (1984) 2nd ed., The Pierce Chemical Co., Rockford, IL).

### EXAMPLE 3

#### 30 Cleavage and Activation Studies of the Recombinant Protease-Activated Receptor 3

PAR3 was demonstrated to be a substrate for thrombin when expressed on the surface of Cos 7 cells (Fig. 6). Human PAR1 or PAR3 cDNAs that were modified to

encode receptors displaying a FLAG epitope (amino acid sequence DYKDDD (SEQ ID NO:12) at a site amino to the thrombin cleavage site were transiently expressed in Cos7 cells. Epitope-tagged PAR1 has been previously described  
5 (Ishii, K. et al. (1993) J. Biol. Chem. 268:9780-9786). The analogous epitope-tagged PAR3 cDNA was constructed so as to encode a new amino terminus with the sequence MDSKGSSQKGSRLLLLLLVSNLLLCQGVVS/DYKDDDDVE-TF (SEQ ID NO:13) representing the prolactin signal peptide,  
10 putative signal peptidase site (/), FLAG epitope DYKDDDD (SEQ ID NO:12) and junction VE fused to amino acid 17 in PAR 3.

cDNAs were subcloned into the mammalian expression vector pBJ1. For receptor cleavage studies Cos 7 cells  
15 were transfected using DEAE-dextran and thrombin-mediated loss of M1 antibody (Kodak) binding to the FLAG epitope of the cell surface using a procedure described by Ishii et al. (Ishii, K. et al. (1993) *supra*). Over 95% of M1 antibody binding was transfection-dependent in this  
20 system. Cells were incubated for 5 min. at 37°C in the presence (open columns) or absence (closed columns) of 20nM thrombin (Fig. 6). For biochemical identification of the cleavage site, cleavage of soluble PAR3 amino terminal exodomain by thrombin was assayed as follows. A  
25 recombinant PAR3 soluble exodomain was prepared in which the amino terminal exodomain residues 21-94 were sandwiched between a translational start and hexahistidine tag (i.e. MG-[PAR3 21-94]-VEHHHHHH; where VEHHHHHH is SEQ ID NO:18). The recombinant protein was  
30 expressed as a soluble polypeptide in *E. coli*, purified, and analyzed before and after thrombin cleavage as previously described for the analogous region of PAR1 (Ishii, K. (1995) J. Biol. Chem. 270:16435-16440).  
Recombinant soluble amino terminal exodomain was cleaved  
35 in solution with 50nM thrombin for 1h at 37°C, then

analyzed by SDS-PAGE. Even prolonged incubation with a high concentration of thrombin yielded only one detectable cleavage event indicating that only one thrombin cleavage site exists in the PAR3 exodomain.

- 5 Amino acid sequencing of the cleavage products revealed only a single new amino terminus with the sequence TFRG (see Fig. 1b). Thus, thrombin recognizes and cleaves PAR3 in the amino terminal exodomain between amino acids K38 and T39 with high specificity.

10

#### EXAMPLE 4

##### PAR3 Signaling Activity

- The ability of PAR3 to mediate signaling by thrombin was tested. *Xenopus* oocytes were microinjected with cRNA encoding epitope-tagged human PAR3 (hPAR3),  
15 hPAR3 bearing the T39P cleavage site mutation, or the F40A tethered ligand domain mutation. Thrombin-triggered <sup>45</sup>Ca release was measured as described in Vu et al. (Vu, T.-K. H. et al. (1991) *supra*). Surface expression of wild type and mutant receptors was confirmed by M1  
20 antibody binding by the method of Ishii, K. et al. (Ishii, K. et al. (1995) J. Biol. Chem. 270:16435-16440; and Ishii, K. et al. (1993) J. Biol. Chem. 268:9780-9786, which references are herein incorporated by reference in their entirety).

- 25 Microinjection of *Xenopus* oocytes with human PAR3 cRNA conferred thrombin-dependent <sup>45</sup>Ca mobilization (Fig. 7) which reflects agonist-triggered phosphoinositide hydrolysis in this system. Mutation of PAR3's thrombin cleavage site ablated thrombin signaling  
30 and thrombin rendered proteolytically inactive by the active site inhibitor PPACK failed to activate PAR3 even at concentrations as high as 1μM. These data strongly

suggest that cleavage of the K 38 -T 39 peptide bond is necessary for PAR3 activation by thrombin.

The specificity of PAR3 and PAR1 signaling was also examined. Protease-triggered  $^{45}\text{Ca}$  release was  
5 measured in *Xenopus* oocytes expressing human PAR1 or PAR3 stimulated with various concentrations of the arginine/lysine specific serine proteases trypsin, Factor Xa, Factor VIIa, tissue plasminogen activator, or plasmin. Chymotrypsin, elastase, and cathepsin G were  
10 also tested. PAR3 was at least as specific for thrombin as thrombin receptor PAR1 (Fig. 10).

PAR3 signaling in Cos 7 cells was also examined. Cos 7 cells were transfected with human PAR1 or PAR3. Cells were then metabolically labelled with  $^3\text{H}$ -inositol  
15 and phosphoinositide hydrolysis was measured in response to the indicated concentrations of  $\alpha$ -thrombin (Fig. 8) or  $\gamma$ -thrombin (Fig. 9) as described by Ishii, et al. and Nanevicz et al. (Ishii, K. et al. (1993) *supra*; and Nanevicz, T. et al. (1996) *J. Biol. Chem.* 271:702-706).

20 Co-transfection with  $\alpha 16$ , a G protein  $\alpha$ -subunit expressed in hematopoietic cell lines (Amatruda III, T.T. et al. (1991) *J. Biol. Chem.* 266:5587-5591) caused a 50-150% increase in the maximal PAR3-mediated response to thrombin in these cells in each of three separate  
25 experiments (Fig. 7).

The  $\text{EC}_{50}$  for thrombin signaling through PAR3 in this system was approximately 0.2 nM, comparable to that seen with PAR1 and well within physiologically achievable thrombin concentrations (Fig 8).  $\gamma$ -thrombin, which is  
30 defective in its anion-binding exosite (Rydel, T.J. et al. (1994) *J. Biol. Chem.* 269:22000-22006), was two log units less potent than  $\alpha$ -thrombin ( $\text{EC}_{50}$  = 20nM; Fig. 9). Similarly, incubation of  $\alpha$ -thrombin with the fibrinogen binding exosite blocker hirugen (Skrzypczak, J.E. et al.  
35 (1991) *J. Mol. Biol.* 221:1379-1393) right-shifted the

dose response curve two logs (not shown). Alanine substitution at F 48 and E 49 in PAR3's hirudin-like sequence, residues predicted to dock with thrombin's fibrinogen-binding exosite by analogy with hirudin and PAR1 (Fig. 5B) also caused a decrease in thrombin signaling by PAR3. These data strongly suggest that PAR3 interacts with thrombin in a manner similar to PAR1 (Mathews, I. I., et al. (1994) *Biochem.* 33:3266-3279). Specifically, it is likely that PAR3 amino acids 48-52 (FEEFP, SEQ ID NO:14) dock with thrombin's fibrinogen-binding exosite while amino acids 35-38 (LTPK, SEQ ID NO:15) dock with thrombin's active center leading to cleavage of the K 38 - T 39 peptide bond.

Synthetic peptides that mimic the new amino terminus unmasked by receptor proteolysis, the so called "tethered ligand domain", act as agonists for PAR1 and PAR2 (Vu, T. K.-H. et al. (1991) *Cell* 64:1057-1068; Nystedt, S. et al. (1994) *PNAS USA* 91:9208-9212; and USPN 5,256,766, which references are herein incorporated by reference in their entirety).

Peptides homologous to the tethered domain of PAR3 may be tested as potential agonists of PAR3 activity. Two peptides, TFRGAP (SEQ ID NO:16) and TFRGAPPNS (SEQ ID NO:17) were synthesized and tested for their ability to mimic the action of thrombin by causing PAR3 signaling as measured by phosphoinositide hydrolysis. Cos 7 cells expressing human PAR3 were incubated with the peptides at concentrations up to 100 $\mu$ M. Phosphoinositide hydrolysis was not observed to be above control levels indicating that the synthetic peptides caused no detectable signaling by PAR3 under these conditions, whereas an EC<sub>50</sub> of 0.2 nM was determined for  $\alpha$ -thrombin under the same assay conditions. These results demonstrate that monitoring phosphoinositide hydrolysis provides a useful

means for assessing potential agonists for activity on PAR3 signaling for use as potential pharmaceuticals.

The tethered ligand domain of PAR3 was required for PAR3 activation by thrombin. Substitution of Ala for  
5 Phe 40 (the F40A PAR3 mutant), which is analogous to the critical Phe 43 in PAR1's tethered ligand (Scarborough, R.M. et al. (1992) J. Biol. Chem. 267:13146-13149), ablated PAR3 signaling but not PAR3 cleavage by thrombin. The observation that cleavage of the Lys 38-Thr 39  
10 peptide bond is necessary for PAR3 activation suggests that PAR3 is probably activated by the same tethered ligand mechanism utilized by PAR1 and 2.

#### EXAMPLE 5

##### PAR3 Tissue Expression in Mouse and Human

15 In situ hybridization of mouse tissue revealed the presence of PAR3 mRNA in megakaryocytes in mouse spleen. In the tissues examined (brain, eye, thymus, heart, lung, liver spleen, pancreas, stomach, small intestine, colon, kidneys, bladder, uterus, ovary, testis, skeletal  
20 muscle, peripheral nerve, and skin), megakaryocytes in the spleen were the only cells which displayed clearcut hybridization over background. Control samples in which hybridization was performed with a sense strand probe control were negative for all cells. Northern analysis  
25 of mouse tissues for PAR3 mRNA showed signals in spleen and lung, with low levels seen in brain, heart, and other tissues. Spleen is a hematopoietic organ in mouse, and megakaryocytes are sometimes seen trapped in the pulmonary microvasculature. Thus both Northern and in  
30 situ hybridization data suggest that PAR3 is most abundantly expressed in megakaryocytes in the mouse.

The pharmacology of hPAR3 activation in Cos cells resembles that of mouse platelet activation. Both responses show subnanomolar EC<sub>50</sub>s for activation by  $\alpha$ -thrombin and are thrombin active site- and fibrinogen-binding exosite-dependent. These observations support the concept that the mouse homolog of PAR3 is a thrombin receptor that mediates thrombin responses in mouse platelets. Whether human PAR3 function in human platelets remains to be determined.

10       The *in situ* hybridization studies were performed as follows. Anesthetized adult C57BL/6 mice were perfusion-fixed with 4% paraformaldehyde. Organs to be tested were dissected, trimmed, and immersion-fixed for 4 hours in 4% paraformaldehyde. Processed tissues were  
15 embedded in paraffin, and 5 mm sections were cut. Sense or antisense <sup>35</sup>S-riboprobe was transcribed *in vitro* from mouse PAR2 cDNA subcloned into the *Eco*R1 site of pBluescript II SK<sup>-</sup> (Stratagene, San Diego, CA). Hybridization, wash, and development conditions were as  
20 reported for mouse PAR1 (Soifer, S.J. et al. (1993) Am. J. Pathol. 144:60-69). To carry out Northern analysis a <sup>32</sup>P-labeled probe for the mouse message was generated by random priming (Prime-It II kit; Stratagene) of PCR-amplified DNA fragments corresponding to mouse cDNA  
25 codons representing transmembrane domains 2 to 3. High stringency hybridizations and washes were performed as per the Clontech protocol for Northern analysis.

Northern analysis of human tissues revealed that PAR3 mRNA is widely distributed with signals noted in  
30 small intestine, bone marrow, heart, pancreas, lung, liver, adrenal, trachea, lymph node, stomach, and peripheral blood leukocytes. The role of PAR3 in these various human tissues awaits definition; the finding of PAR3 in human bone marrow and leukocytes is consistent



with PAR3's playing a role in mediating activation of platelets and other hematopoietic cells by thrombin.

#### EXAMPLE 6

##### Assays for Protease-Activated Receptor 3 Function

5           Useful receptor fragments or analogs of the invention are those which interact with thrombin and are activated to initiate the cascade of events associated with thrombin:receptor interaction. Such an interaction may be detected by an *in vitro* functional assay method  
10 (e.g., the phosphoinositide hydrolysis assay, <sup>45</sup>Ca efflux assay, or platelet aggregation assay described herein). This method includes, as components, thrombin and a recombinant protease-activated receptor 3 (or a suitable fragment or analog) configured to permit thrombin binding  
15 (e.g., those polypeptides described herein). Thrombin may be obtained from Sigma Chemical Co. (St. Louis, MO) or similar supplier.

          Preferably, the protease-activated receptor 3 component is produced by a cell that naturally presents  
20 substantially no receptor on its surface, e.g., by engineering such a cell to contain nucleic acid encoding the receptor component in an appropriate expression system. Suitable cells are, e.g., those discussed above with respect to the production of recombinant receptor,  
25 such as Rat 1 cells or COS-7 cells.

#### EXAMPLE 7

##### Screening For Protease-Activated Receptor 3 Activator Antagonists and Agonists Antagonists

30           As discussed above, one aspect of the invention features screening for compounds that inhibit the interaction between thrombin (or other PAR3 activating

compound) and the protease-activated receptor 3, thereby preventing or reducing the cascade of events that are mediated by that interaction. The elements of the screen are a PAR3 activator (such as thrombin), a candidate  
5 antagonist, and recombinant PAR3 (or a suitable receptor fragment or analog, as outlined above) configured to permit detection of PAR3 activator, antagonist, and PAR3 function. An additional element may be a downstream substrate, such as phosphoinositide, the hydrolysis of  
10 which is used to measure thrombin activity (Ishii, K. et al. (1993) *supra*; and Nanevycz, T. et al. (1996) *supra*).

Inhibition of thrombin-induced platelet aggregation may also be used as a means of monitoring an antagonist of PAR3 receptor activation. Thrombin is  
15 incubated with the candidate inhibitory compound (such as a peptide) for 5 minutes, then the mixture is added to washed platelets and platelet activation is followed as platelet ATP secretion by lumiaggregometry (see, for example, Connolly, A.J. et al. Nature 381:516-519 (1996);  
20 and USPN 5,256,766). Alternately, platelets are incubated with a candidate PAR 3 antagonist for 5 minutes. Thereafter the response to thrombin is measured.

Inclusion of potential antagonists in the  
25 screening assay along with thrombin allows for the screening and identification of authentic receptor antagonists as those which decrease thrombin-mediated events, such as platelet aggregation.

Appropriate candidate thrombin antagonists include  
30 PAR3 fragments, particularly, fragments of the protein predicted to be extracellular and therefore likely to bind thrombin or the tethered ligand; such fragments would preferably include five or more amino acids.

Candidate PAR 3 antagonists include thrombin analogs as well as other peptide and non-peptide compounds and anti-PAR3 antibodies.

#### AGONISTS

5           Another aspect of the invention features screening for compounds that act as PAR3 ligand agonists. Activation of the PAR3 with thrombin or an agonist leads to a cascade of events (such as phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, and platelet aggregation),  
10 providing a convenient means for measuring thrombin or other agonist activity.

          The agonist screening assay of the invention utilizes recombinant cells expressing recombinant PAR3 (or a suitable receptor fragment or analog, as outlined  
15 herein) configured to permit detection of PAR3 function. Alternatively, a cell such as a leukocyte, a platelet, or a mesenchymal cell that naturally expresses PAR3 may be used. Other elements of the screen include a detectable downstream substrate of the PAR3 activation, such as  
20 radiolabelled phosphoinositide, the hydrolysis of which to a detectable product indicates PAR3 activation by the candidate agonist.

$^{45}\text{Ca}$  efflux from a cell expressing PAR3 may be used as a means of measuring receptor activation by  
25 candidate agonists (Williams, J.A. et al., (1988) PNAS USA 85:4939-4943; Vu, T.-K. H., et al. (1991) Cell 64:1057-1068; and USPN 5,256,766, which references are herein incorporated by reference in their entirety).

$^{45}\text{Ca}$  release by oocytes expressing cRNA encoding PAR3 are  
30 assessed as follows. Briefly, intracellular calcium pools are labeled by incubating groups of 30 oocytes in 300  $\mu\text{l}$  calcium-free MBSH containing 50  $\mu\text{Ci}$   $^{45}\text{CaCl}_2$  (10-40 mCi/mg Ca; Amersham) for 4 hours at room temperature.

The labeled oocytes are washed, then incubated in MBSH II without antibiotics for 90 minutes. Groups of 5 oocytes are selected and placed in individual wells in a 24-well tissue culture plate (Falcon 3047) containing 0.5 ml/well  
5 MBSH II without antibiotics. This medium is removed and replaced with fresh medium every 10 minutes, the harvested medium is analyzed by scintillation counting to determine  $^{45}\text{Ca}$  released by the oocytes during each 10-minute incubation. The 10-minute incubations are  
10 continued until a stable baseline of  $^{45}\text{Ca}$  release per unit time is achieved. Two additional 10-minute collections are obtained, then test medium including agonist is added and agonist-induced  $^{45}\text{Ca}$  release determined.

A voltage clamp assay provides an alternative  
15 method of monitoring agonist activity. Agonist-induced inward chloride currents are measured in voltage-clamped oocytes expressing thrombin receptor encoding cRNA essentially as previously described (Julius, D. et al. Science (1988) 241:558-563, herein incorporated by  
20 reference in its entirety) except that the single electrode voltage-clamp technique is employed.

Platelet aggregation may also be used as a means of monitoring PAR3 receptor activation (see, for example, Connolly, A.J. et al. Nature 381:516-519 (1996). In  
25 particular, mouse platelets may utilize only PAR 3 for thrombin signaling. Human platelets may use both PAR 1 and PAR 3. Thus both would be useful in deleting against function at PAR 3.

An agonist useful in the invention is one which imitates the normal thrombin-mediated signal transduction pathway leading, e.g., to an increase in phosphoinositide hydrolysis. Appropriate candidate agonists include  
5 thrombin analogs or PAR3 tethered ligand domains or other agents which mimic the action of thrombin or the PAR 3 tethered ligand domain. Agonists would be useful for aiding discovery of antagonists.

#### EXAMPLE 8

##### 10 Anti-Protease-Activated Receptor 3 Antibodies

Protease-activated receptor 3 (or immunogenic receptor fragments or analogs) may be used to raise antibodies useful in the invention. Receptor fragments preferred for the production of antibodies are those  
15 fragments deduced or shown experimentally to be extracellular.

Antibodies directed to PAR3 peptides are produced as follows. Peptides corresponding to all or part of the PAR3 protein are produced using a peptide synthesizer by  
20 standard techniques. The peptides are coupled to KLH with m-maleimide benzoic acid N-hydroxysuccinimide ester. The KLH-peptide is mixed with Freund's adjuvant and injected into animals, e.g. guinea pigs or goats, to produce polyclonal antibodies. Monoclonal antibodies may  
25 be prepared using the PAR3 polypeptides described above and standard hybridoma technology (see, e.g., Kohler et al., Nature (1975) 256:495, 1975; Kohler et al., Eur. J. Immunol. (1976) 6:292; Kohler et al., Eur. J. Immunol. (1976) 6:511; Hammerling et al., in Monoclonal Antibodies  
30 and T Cell Hybridomas, Elsevier, NY, (1981); and Ausubel et al., supra). Antibodies are purified by peptide antigen affinity chromatography.

Once produced, antibodies are tested for their ability to bind PAR3 by specific binding to the surface of PAR3-transfected cells by Western blot or immunoprecipitation analysis (such as by the methods  
5 described in Ausubel et al., supra).

Antibodies which specifically recognize PAR3 are considered to be likely candidates for useful antagonists; such candidates are further tested for their ability to specifically interfere with the interaction  
10 between thrombin and PAR3 (using the functional antagonist assays described herein). Antibodies which antagonize thrombin:PAR3 binding or PAR3 function are considered to be useful antagonists in the invention.

#### EXAMPLE 9

#### 15 THERAPY

Particularly suitable therapeutics for the treatment of wound healing, thrombosis, atherosclerosis, restenosis, inflammation, and other thrombin-mediated signalling disorders are the agonists and antagonists  
20 described above formulated in an appropriate buffer such as physiological saline. Where it is particularly desirable to mimic a receptor fragment conformation at the membrane interface, the fragment may include a sufficient number of adjacent transmembrane residues. In  
25 this case, the fragment may be associated with an appropriate lipid fraction (e.g., in lipid vesicles or attached to fragments obtained by disrupting a cell membrane). Alternatively, anti-PAR3 antibodies produced as described above may be used as a therapeutic. Again,  
30 the antibodies would be administered in a pharmaceutically-acceptable buffer (e.g., physiological saline). If appropriate, the antibody preparation may be combined with a suitable adjuvant.

Antibodies to PAR 3 are useful antagonists which can be formulated as indicated above. Other therapeutically useful antagonists are peptides derived from PAR3 that bind to and block thrombin and include  
5 formulation comprising a pharmaceutically acceptable carrier and one or more of the following:

- (1) the isolated sequence  
LPIKTFRGAPPNSFEEFPFSALE;
- 10 (2) uncleavable thrombin inhibitor  
LPIKPFRGAPPNSFEEFPFSALE where the PAR 3 cleavage site P1' is mutated to block cleavage;
- 15 (3) uncleavable thrombin inhibitor LPI  
(hR)TFRGAPPNSFEEFPFSALE where the PAR 3 cleavage site P1 is mutated to block cleavage;  
hR is beta-homoarginine (the extra methylene group is in the main chain);
- 20 (4) uncleavable thrombin inhibitor  
(dF)PRPFRGAPPNSFEEFPFSALE where the good active site binding sequence dFPR is substituted for LPIK; dF is D-Phenylalanine;
- 25 (5) any of (1)-(4) above where all or part of the sequence TFRGAPPNS is replaced with spacer sequences such as GGG;
- (6) variations and combinations of (1)-(5) which act as antagonists.

The therapeutic preparation is administered in accordance with the condition to be treated. Ordinarily,  
30 it will be administered intravenously, at a dosage, of a duration, and with the appropriate timing to elicit the desired response. Appropriate timing refers to, for example, time relative to wounding, time intervals between therapeutic administrations, and the like, at

which administration of therapeutic preparation elicits the desired response. Alternatively, it may be convenient to administer the therapeutic orally, nasally, or topically, e.g., as a liquid or a spray. The dosages  
5 are determined to be an amount of the therapeutic agent delivered to an animal that substantially reduces or alleviates disease symptoms. Treatment may be repeated as necessary for substantial reduction or alleviation of disease symptoms.

10 PAR3 activator agonists can be used for the treatment of bleeding. Antagonists may be useful in controlling the formation of clots that cause heart attack and stroke, mediating inflammation and the proliferative responses to injury in normal wound healing  
15 and a variety of diseases including atherosclerosis, restenosis, pulmonary inflammations (ARDS), glomerulosclerosis, etc.

The methods of the invention may be used to screen therapeutic receptor activator agonists and antagonists  
20 for their effectiveness in altering thrombin-mediated biological events, such as phosphoinositide hydrolysis or other cell signalling events by the assays described above. Where a non-human mammal is treated or where a therapeutic for a non-human animal is screened, the PAR3  
25 or receptor fragment or analog or the antibody employed is preferably specific for that species.

#### OTHER EMBODIMENTS

Polypeptides according to the invention include any protease-activated receptors (as described herein).  
30 Such receptors may be derived from any source, but are preferably derived from a vertebrate animal, e.g., a human or mouse. These polypeptides are used, e.g., to



screen for antagonists which disrupt, or agonists which mimic, a thrombin:receptor interaction.

Polypeptides of the invention also include any analog or fragment of a PAR3 capable of interacting with  
5 thrombin. Such analogs and fragments may also be used to screen for PAR3 ligand antagonists or agonists. In addition, that subset of receptor fragments or analogs which bind thrombin and are, preferably, soluble (or insoluble and formulated in a lipid vesicle) may be used  
10 as antagonists to reduce the *in vivo* concentration of endogenous thrombin, either circulating concentration or local concentration. The efficacy of a receptor analog or fragment is dependent upon its ability to interact with thrombin; such an interaction may be readily assayed  
15 using PAR3 functional assays (e.g., those described herein).

Specific receptor analogs of interest include full-length or partial receptor proteins including an amino acid sequence which differs only by conservative  
20 amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for leucine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the  
25 amino acid sequence which do not destroy the receptors' ability to signal thrombin-mediated events (e.g., as assayed above).

Specific receptor fragments of interest include any portion of the PAR3 which is capable of interacting  
30 with thrombin, for example, all or part of the extracellular domains predicted from the deduced amino acid sequence. Such fragments may be useful as antagonists (as described above), and are also useful as immunogens for producing antibodies which neutralize the  
35 activity of PAR3 *in vivo* (e.g., by interfering with the

interaction between the receptor and thrombin). The sequence of figure 5B is most likely to bind thrombin. Modification of the (K38/T39) cleavage site for example, substitution of proline for T39 will render peptides  
5 mimicking this site uncleavable. Such peptides will bind thrombin with high affinity.

Extracellular regions of novel protease-activated receptors may be identified by comparison with related proteins of similar structure (e.g., other members of the  
10 G-protein-coupled receptor family); useful regions are those exhibiting homology to the extracellular domains of well-characterized members of the family.

Alternatively, from the primary amino acid sequence, the secondary protein structure and, therefore,  
15 the extracellular domain regions may be deduced semi-empirically using a hydrophobicity/hydrophilicity calculation such as the Chou-Fasman method (see, e.g., Chou and Fasman, Ann. Rev. Biochem. (1978) 47:251). Hydrophilic domains, particularly ones surrounded by  
20 hydrophobic stretches (e.g., transmembrane domains) present themselves as strong candidates for extracellular domains. Finally, extracellular domains may be identified experimentally using standard enzymatic digest analysis, e.g., tryptic digest analysis.

25 Candidate fragments (e.g., any extracellular fragment) are tested for interaction with thrombin by the assays described herein (e.g., the assay described above). Such fragments are also tested for their ability to antagonize the interaction between thrombin and its  
30 endogenous receptor, such as PAR3, using the assays described herein. Analogs of useful receptor fragments (as described above) may also be produced and tested for efficacy as screening components or antagonists (using the assays described herein); such analogs are also  
35 considered to be useful in the invention.

Identification of the receptor(s) that mediate thrombin signaling provides potential targets for the development of drugs that block thrombin's undesirable actions or mimic its desirable activities. Thrombin  
5 receptor antagonists may be used for inhibition of platelet-dependent thrombosis in the setting of unstable angina and myocardial infarction or for blocking thrombin's proinflammatory actions on endothelial cells in the setting of vascular injury. Thrombin receptor  
10 agonists may be used to promote hemostasis and fibroblast proliferation at wound sites.

Unmasked tethered ligand domain peptides may provide lead structures for the development of PAR3 agonists or antagonists.

15 The instant invention is shown and described herein in what is considered to be the most practical, and preferred embodiments. It is recognized, however, that departures may be made therefrom, which are within the scope of the invention, and that obvious  
20 modifications will occur to one skilled in the art upon reading this disclosure.

CLAIMS

That which is claimed is:

1. Substantially pure DNA encoding a protease-activated receptor 3.
- 5           2. The DNA of claim 1, wherein the DNA is mammalian.
3. Substantially pure DNA having the nucleotide sequence selected from the group consisting of Fig. 1 (SEQ ID NO:1), or degenerate variants thereof, and  
10 encoding the amino acid sequence of Fig. 1 (SEQ ID NO:3); Fig. 2 (SEQ ID NO:2), or degenerate variants thereof encoding an amino acid sequence comprising the amino acid sequence of Fig. 1 (SEQ ID NO:3); Fig. 3 (SEQ ID NO:4), or degenerate variants thereof encoding the amino acid  
15 sequence of Fig. 2 (SEQ ID NO:6); and Fig. 4 (SEQ ID NO:5), or degenerate variants thereof encoding an amino acid sequence comprising the amino acid sequence of Fig. 3 (SEQ ID NO:6).
4. Substantially pure DNA having 50% or greater  
20 sequence identity to the DNA sequence of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5 and which hybridizes to the DNA sequence SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:5, respectively.
- 25           5. An isolated protease-activated receptor 3 protein.

6. The substantially pure protein of claim 5 having an amino acid sequence selected from the group consisting of the sequence shown in Fig. 1 (SEQ ID NO:3). and the sequence shown in Fig. 2 (SEQ ID NO:6).

5 7. A substantially pure polypeptide having an amino acid sequence which is at least 80% identical to an amino acid sequence selected from the group consisting of the sequence shown in Fig. 1 (SEQ ID NO:3) and the sequence shown in Fig. 2 (SEQ ID NO:6), wherein

- 10 a) said polypeptide is activated by thrombin; and  
b) said polypeptide mediates phosphoinositide hydrolysis in a cell expressing said polypeptide on its surface.

8. A substantially pure polypeptide which is a  
15 fragment or analog of a protease-activated receptor 3 comprising a domain capable of activation by thrombin and mediating phosphoinositide hydrolysis.

9. A vector comprising the DNA of claim 1.

10. A cell comprising the vector of claim 9.

20 11. An assay device, comprising:  
a support surface;  
and a cell of claim 10.

12. The assay device of claim 11, wherein the  
cell is bound to the support surface or present in a  
25 suspension on the support surface.

13. A method of testing a candidate compound for its ability to act as an agonist of a protease-activated receptor 3 ligand, the method comprising:

- a) contacting a candidate compound with a cell  
5 which expresses on its surface a recombinant protease-activated receptor 3 protein or biologically active fragment or analog thereof;
- b) measuring PAR3-mediated response of the cell;  
and
- 10 c) identifying the candidate compound as an agonist wherein the contacting causes a substantial increase in PAR3-mediated response.

14. A method of testing a candidate compound for the ability to act as an antagonist of a protease-  
15 activated receptor 3 ligand, the method comprising:

- a) contacting in the presence of a protease-activated receptor agonist a candidate compound with a cell which expresses on its surface a recombinant protease-activated receptor 3 protein or biologically  
20 active fragment or analog thereof;
- b) measuring PAR3-mediated response of the cell;  
and
- c) identifying the candidate compound as an antagonist wherein the contacting causes a substantial  
25 decrease in PAR3-mediated response relative to PAR3-mediated response in the absence of the candidate antagonist.

15. The method of claim 14, wherein the cell is a mammalian cell which normally presents substantially no  
30 protease-activated receptor 3 on its surface, the PAR3-mediated response measured in intracellular phosphoinositide hydrolysis in the cell.

16. The method of claim 14, further comprising:  
mixing thrombin with platelets and the identified  
candidate compound; and

observing the effect of the candidate compound on  
5 mediating platelet aggregation.

17. A therapeutic composition, comprising:  
a protease-activated receptor 3 ligand agonist;

and

a physiologically-acceptable carrier.

10 18. A therapeutic composition, comprising:  
a protease-activated receptor 3 ligand antagonist;

and

a physiologically-acceptable carrier.

19. The composition of claim 18, wherein the antagonist is selected from the group consisting of:

- (1) the isolated sequence  
LPIKTFRGAPPNSFEEFPFSALE;
- 5 (2) uncleavable thrombin inhibitor  
LPIKPFRGAPPNSFEEFPFSALE where the PAR 3  
cleavage site P1' is muted to block cleavage;
- (3) uncleavable thrombin inhibitor LP|  
10 (hR)TFRGAPPNSFEEFPFSALE where the PAR 3  
cleavage site P1 is mutated to block  
cleavage;  
hR is beta-homoarginine (the extra methylene  
group is in the main chain);
- 15 (4) uncleavable thrombin inhibitor  
(dF)PRPFRGAPPNSFEEFPFSALE where the good  
active site binding sequence dFPR is  
substituted for LPIK; dF is D-Phenylalanine;
- (5) any of (1)-(4) above where all or part of the  
20 sequence TFRGAPPNS is replaced with spacer  
sequences such as GGG;
- (6) variations and combinations of (1)-(5) which  
act as antagonists.

20. A method of treatment, comprising:  
administering to a patient a therapeutically  
25 effective amount of the composition of claim 18.



PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF

Abstract of the Disclosure

Disclosed are cDNAs and genomic DNAs encoding  
5 protease-activated receptor 3 (PAR3) from mouse and  
human, and the recombinant polypeptides expressed from  
such cDNAs. The recombinant receptor polypeptides,  
receptor fragments and analogs expressed on the surface  
of cells are used in methods of screening candidate  
10 compounds for their ability to act as agonists or  
antagonists to the effects of interaction between  
thrombin and PAR3. Agonists are used as therapeutics to  
treat wounds, thrombosis, atherosclerosis, restenosis,  
inflammation, and other thrombin-activated disorders.  
15 Antagonists are used as therapeutics to control blood  
coagulation and thereby treating heart attack and stroke.  
Antagonists mediate inflammatory and proliferative  
responses to injury as occur in normal wound healing and  
variety of diseases including atherosclerosis,  
20 restenosis, pulmonary inflammation (ARDS) and  
glomerulosclerosis. Antibodies specific for a protease-  
activated receptor 3 (or receptor fragment or analog) and  
their use as a therapeutic are also disclosed.

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

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Kimberly W. Zuehlke  
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Shaun R. Coughlin et al.

Serial No.: Unassigned (Divisional of USSN 08/742,440)

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Title: PROTEASE-ACTIVATED RECEPTOR 3 AND  
USES THEREOF

SUBMISSION OF FORMAL DRAWINGS

Assistant Commissioner for Patents  
Washington, D.C. 20231  
**Attention: Official Draftsman**

Sir:

Enclosed are 16 sheets of formal drawings in connection with the above identified case.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 18-0580.

Respectfully submitted,

By: Dianna L. DeVore  
Dianna L. DeVore  
Registration No. 42,484

BOZICEVIC & REED LLP  
285 Hamilton Avenue, Suite 200  
Palo Alto, California 94301  
Telephone: (650) 327-3400  
Facsimile: (650) 327-3231

FIG. 1-1

			10				20				30				40				50
	*		*		*		*		*		*		*		*		*		*
TG	ACT	TTG	TAT	ACT	TAA	CAA	CAT	CCT	GTA	GCC	GGG	TCT	CAG	GAC	ATC	AAG			
AC	TGA	AAC	ATA	TGA	ATT	GTT	GTA	GGA	CAT	CGG	CCC	AGA	GTC	CTG	TAG	TTC			
	T	L	Y	T	*	Q	H	P	V	A	G	S	Q	D	I	K>			
			60				70				80				90				
	*		*		*		*		*		*		*		*		*		
ATG	AAA	ATC	CTT	ATC	TTG	GTT	GCA	GCT	GGG	CTG	CTG	TTT	CTG	CCA	GTC				
TAC	TTT	TAG	GAA	TAG	AAC	CAA	CGT	CGA	CCC	GAC	GAC	AAA	GAC	GGT	CAG				
M	K	I	L	I	L	V	A	A	G	L	L	F	L	P	V>				
100			110				120				130				140				
	*		*		*		*		*		*		*		*		*		
ACT	GTT	TGC	CAA	AGT	GGC	ATA	AAT	GTT	TCA	GAC	AAC	TCA	GCA	AAG	CCA				
TGA	CAA	ACG	GTT	TCA	CCG	TAT	TTA	CAA	AGT	CTG	TTG	AGT	CGT	TTC	GGT				
	T	V	C	Q	S	G	I	N	V	S	D	N	S	A	K	P>			
	150			160			170				180				190				
	*		*		*		*		*		*		*		*		*		
ACC	TTA	ACT	ATT	AAG	AGT	TTT	AAT	GGG	GGT	CCC	CAA	AAT	ACC	TTT	GAA				
TGG	AAT	TGA	TAA	TTC	TCA	AAA	TTA	CCC	CCA	GGG	GTT	TTA	TGG	AAA	CTT				
	T	L	T	I	K	S	F	N	G	G	P	Q	N	T	F	E>			
	200			210			220				230				240				
	*		*		*		*		*		*		*		*		*		
GAA	TTC	CCA	CTT	TCT	GAC	ATA	GAG	GGC	TGG	ACA	GGA	GCC	ACC	ACA	ACT				
CTT	AAG	GGT	GAA	AGA	CTG	TAT	CTC	CCG	ACC	TGT	CCT	CGG	TGG	TGT	TGA				
	E	F	P	L	S	D	I	E	G	W	T	G	A	T	T	T>			
	250			260			270				280				290				
	*		*		*		*		*		*		*		*		*		
ATA	AAA	GCG	GAG	TGT	CCC	GAG	GAC	AGT	ATT	TCA	ACT	CTC	CAC	GTG	AAT				
TAT	TTT	CGC	CTC	ACA	GGG	CTC	CTG	TCA	TAA	AGT	TGA	GAG	GTG	CAC	TTA				
	I	K	A	E	C	P	E	D	S	I	S	T	L	H	V	N>			
	300			310			320				330								
	*		*		*		*		*		*		*		*		*		
AAT	GCT	ACC	ATA	GGA	TAC	CTG	AGA	AGT	TCC	TTA	AGT	ACC	CAA	GTG	ATA				
TTA	CGA	TGG	TAT	CCT	ATG	GAC	TCT	TCA	AGG	AAT	TCA	TGG	GTT	CAC	TAT				
	N	A	T	I	G	Y	L	R	S	S	L	S	T	Q	V	I>			
340			350				360				370				380				
	*		*		*		*		*		*		*		*		*		
CCT	GCC	ATC	TAT	ATC	CTG	CTG	TTT	GTG	GTT	GGT	GTA	CCA	TCC	AAC	ATC				
GGA	CGG	TAG	ATA	TAG	GAC	GAC	AAA	CAC	CAA	CCA	CAT	GGT	AGG	TTG	TAG				
	P	A	I	Y	I	L	L	F	V	V	G	V	P	S	N	I>			
	390			400			410				420				430				
	*		*		*		*		*		*		*		*		*		
GTG	ACC	CTG	TGG	AAA	CTC	TCC	TTA	AGG	ACC	AAA	TCC	ATC	AGT	CTG	GTC				
CAC	TGG	GAC	ACC	TTT	GAG	AGG	AAT	TCC	TGG	TTT	AGG	TAG	TCA	GAC	CAG				
	V	T	L	W	K	L	S	L	R	T	K	S	I	S	L	V>			

FIG. 1-2

```

      440      450      460      470      480
*   *   *   *   *   *   *   *   *   *
ATC TTT CAC ACC AAC CTG GCC ATC GCA GAT CTC CTT TTC TGT GTC ACA
TAG AAA GTG TGG TTG GAC CGG TAG CGT CTA GAG GAA AAG ACA CAG TGT
I   F   H   T   M   L   A   I   A   D   L   L   F   C   V   T>

      490      500      510      520      530
*   *   *   *   *   *   *   *   *   *
CTG CCA TTT AAG ATC GCC TAC CAT CTC AAT GGC AAC AAC TGG GTA TTT
GAC GGT AAA TTC TAG CGG ATG GTA GAG TTA CCG TTG TTG ACC CAT AAA
L   P   F   K   I   A   Y   H   L   N   G   N   N   W   V   F>

      540      550      560      570
*   *   *   *   *   *   *   *   *   *
GGC GAG GTC ATG TGC CGG ATC ACC ACG GTC GTT TTC TAC GGC AAC ATG
CCG CTC CAG TAC ACG GCC TAG TGG TGC CAG CAA AAG ATG CCG TTG TAC
G   E   V   M   C   R   I   T   T   V   V   F   Y   G   N   M>
580      590      600      610      620
*   *   *   *   *   *   *   *   *   *
TAC TGC GCT ATC CTG ATC CTC ACT TGC ATG GGC ATC AAC CGC TAC CTG
ATG ACG CGA TAG GAC TAG GAG TGA ACG TAC CCG TAG TTG GCG ATG GAC
Y   C   A   I   L   I   L   T   C   M   G   I   N   R   Y   L>

      630      640      650      660      670
*   *   *   *   *   *   *   *   *   *
GCC ACG GCT CAC CCT TTC ACA TAC CAG AAG CTG CCC AAA CGC AGC TTC
CGG TGC CGA GTG GGA AAG TGT ATG GTC TTC GAC GGG TTT GCG TCG AAG
A   T   A   H   P   F   T   Y   Q   K   L   P   K   R   S   F>
680      690      700      710      720
*   *   *   *   *   *   *   *   *   *
TCC TTG CTC ATG TGT GGC ATA GTG TGG GTC ATG GTT TTC TTA TAC ATG
AGG AAC GAG TAC ACA CCG TAT CAC ACC CAG TAC CAA AAG AAT ATG TAC
S   L   L   M   C   G   I   V   W   V   M   V   F   L   Y   M>

      730      740      750      760      770
*   *   *   *   *   *   *   *   *   *
CTG CCC TTT GTC ATC CTG AAG CAG GAG TAC CAC CTC GTC CAC TCA GAG
GAC GGG AAA CAG TAG GAC TTC GTC CTC ATG GTG GAG CAG GTG AGT CTC
L   P   F   V   I   L   K   Q   E   Y   H   L   V   H   S   E>

      780      790      800      810
*   *   *   *   *   *   *   *   *   *
ATC ACC ACC TGC CAC GAT GTC GTC GAC GCG TGC GAG TCC CCA TCA TCC
TAG TGG TGG ACG GTG CTA CAG CAG CTG CGC ACG CTC AGG GGT AGT AGG
I   T   T   C   H   D   V   V   D   A   C   E   S   P   S   S>
820      830      840      850      860
*   *   *   *   *   *   *   *   *   *
TTC CGA TTC TAC TAC TTC GTC TCC TTA GCA TTC TTT GGG TTC CTC ATC
AAG GCT AAG ATG ATG AAG CAG AGG AAT CGT AAG AAA CCC AAG GAG TAG
F   R   F   Y   Y   F   V   S   L   A   F   F   G   F   L   I>

```

FIG. 1-3

```

      870      880      890      900      910
      *      *      *      *      *
CCG TTT GTG ATC ATC ATC TTC TGT TAC ACG ACT CTC ATC CAC AAA CTT
GGC AAA CAC TAG TAG TAG AAG ACA ATG TGC TGA GAG TAG GTG TTT GAA
P F V I I I F C Y T T L I H K L>
      920      930      940      950      960
      *      *      *      *      *
AAA TCA AAG GAT CGG ATA TGG CTG GGC TAC ATC AAG GCC GTC CTC CTC
TTT AGT TTC CTA GCC TAT ACC GAC CCG ATG TAG TTC CGG CAG GAG GAG
K S K D R I W L G Y I K A V L L>
      970      980      990      1000      1010
      *      *      *      *      *
ATC CTT GTG ATT TTC ACA ATT TGC TTT GCC CCC ACC AAC ATC ATA CTC
TAG GAA CAC TAA AAG TGT TAA ACG AAA CGG GGG TGG TTG TAG TAT GAG
I L V I F T I C F A P T N I I L>
      1020      1030      1040      1050
      *      *      *      *      *
GTA ATC CAC CAT GCC AAC TAC TAC TAC CAC AAT ACC GAC AGC TTG TAC
CAT TAG GTG GTA CGG TTG ATG ATG ATG GTG TTA TGG CTG TCG AAC ATG
V I H H A N Y Y Y H N T D S L Y>
1060      1070      1080      1090      1100
      *      *      *      *      *
TTT ATG TAT CTT ATT GCT CTG TGC CTG GGG AGC CTG AAT AGC TGC CTA
AAA TAC ATA GAA TAA CGA GAC ACG GAC CCC TCG GAC TTA TCG ACG GAT
F M Y L I A L C L G S L N S C L>
      1100      1120      1130      1140      1150
      *      *      *      *      *
GAT CCA TTC CTT TAC TTT GTC ATG TCG AAA GTT GTA GAT CAG CTT AAT
CTA GGT AAG GAA ATG AAA CAG TAC AGC TTT CAA CAT CTA GTC GAA TTA
D P F L Y F V M S K V V D Q L N>
      1160      1170      1180      1190      1200
      *      *      *      *      *
CCT TAG TCG GCA ATG GCA AGA CCA CTT TAG AGA CCA AGG AGA GAT ATC
GGA ATC AGC CGT TAC CGT TCT GGT GAA ATC TCT GGT TCC TCT CTA TAG
P * S A M A R P L * R P R R D I>
      1210      1220
      *      *      *      *
TGG GAA GAC ATA CAT GCT TGG C
ACC CTT CTG TAT GTA CGA ACC G
W E D I H A W X>

```

FIG. 2-1

10	20	30	40	50
* *	* *	* *	* *	* *
CCATATGCTA	ATATTTCTT	TCAATTACAG	GCATAAATGT	TTCAGACAAC
60	70	80	90	100
* *	* *	* *	* *	* *
TCAGCAAAGC	CAACCTTAAC	TATTAAGAGT	TTTAATGGGG	GTCCCCAAAA
110	120	130	140	150
* *	* *	* *	* *	* *
TACCTTTGAA	GAATTC----	---TACAAC	CTCCATGTGA	ATAATGCTAC
160	170	180	190	200
* *	* *	* *	* *	* *
CATGGGATAC	CTGAGAAGTT	CCTTAAGTAC	CAAAGTGATA	CCTGCCATCT
210	220	230	240	250
* *	* *	* *	* *	* *
ACATCCTGGT	GTTTGTGATT	GGTGTACCAG	CGAACATCGT	GACCCTGTGG
260	270	280	290	300
* *	* *	* *	* *	* *
AAACTCTCCT	CAAGGACCAA	ATCCATCTGT	CTGGTCATCT	TTCACACCAA
310	320	330	340	350
* *	* *	* *	* *	* *
CCTGGCCATC	GCGGATCTCC	TTTTCTGTGT	CACGCTGCCG	TTTAAGATC-
360	370	380	390	400
* *	* *	* *	* *	* *
-CCTACCATC	TCAATGGCAA	CAACTGGGTA	TTTGGCGAGG	TCATGTGCCG
410	420	430	440	450
* *	* *	* *	* *	* *
GATCACCACG	GTCGTTTTCT	ACGGCAACAT	GTACTGCGCT	A---TCCTGA
460	470	480	490	500
* *	* *	* *	* *	* *
TCCTCACCTG	CATGGGCATC	AACCGCTACC	TGGCCACGGC	TCACCCTTTC
510	520	530	540	550
* *	* *	* *	* *	* *
ACATACCAGA	AGCTGCCCAA	ACGCAGCTTC	TCCATGCTCA	TGTGTGGCAT
560	570	580	590	600
* *	* *	* *	* *	* *
GGTGTGGGTC	ATGGTTTTCT	TATACATGCT	GCCCTTTGTC	ATCC---AAG
610	620	630	640	650
* *	* *	* *	* *	* *
CAGGAGTACC	ACCTCGTCCA	CTCCGAGATC	ACCACCTGCC	ACGATGTCGT

FIG. 2-2

660	670	680	690	700
* *	* *	* *	* *	* *
CGACGCGTGC	GANTCCCCAT	CATCCTTCCG	ATTCTACTAC	TTCGTCTCCT
710	720	730	740	750
* *	* *	* *	* *	* *
TAGCATTCTT	TGGGTTCCCTC	ATCCCGTTTG	TGATCATCAT	CTTCTGTTAC
760	770	780	790	800
* *	* *	* *	* *	* *
ACGACTCTCA	TCCACAAACT	TAAATCAAAA	GATCNGATAT	GGCTGGGCTA
810	820	830	840	850
* *	* *	* *	* *	* *
CATCAAGGCC	GTCCTCCTCA	TCCTTGTA	TTTCAACATC	TGCTTCCCCC
860	870	880	890	900
* *	* *	* *	* *	* *
CCACCAAG--	----GATATC	TGGGAAGACG	TACATGCTTG	GCTGACTTGT
910	920	930	940	950
* *	* *	* *	* *	* *
GCATGGCACC	ATCAGCTCAA	TTTTTAATTT	TTTAATTTTA	ATTTAATTTA
960	970	980	990	1000
* *	* *	* *	* *	* *
ATTTTATGTT	TTTGAGACAG	AGCCTCACTG	TGTAGTCCTG	GCTGGCCTGG
1010	1020	1030	1040	1050
* *	* *	* *	* *	* *
CTGGTTCTCT	ATTTAGACCA	GGTTAGCCTT	GAATCACAG	AGATCTGCCT
1060	1070	1080	1090	1100
* *	* *	* *	* *	* *
GCTTCTGCCT	CCCAAGTGCT	GGGTTCAACC	AGGTCTGGCA	AGCGCTCCAT
1110	1120			
* *	* *			
TTTTCAGCTC	CTCTGCAACA	GTGC		

[illegible]

	*		10	*	*		20	*	*		30	*	*		40	*
TGC	TCC	ATG	ATT	TTA	CAG	ATT	TCA	TAA	CGT	TTA	AGA	GAC	GGG	ACT	CAG	
ACG	AGG	TAC	TAA	AAT	GTC	TAA	AGT	ATT	GCA	AAT	TCT	CTG	CCC	TGA	GTC	
C	S	M	I	L	Q	I	S	*	R	L	R	D	G	T	Q>	
50			60				70				80			90		
*		*	*		*		*	*		*		*		*	*	
GTC	ATC	AAA	ATG	AAA	GCC	CTC	ATC	TTT	GCA	GCT	GCT	GGC	CTC	CTG	CTT	
CAG	TAG	TTT	TAC	TTT	CGG	GAG	TAG	AAA	CGT	CGA	CGA	CCG	GAG	GAC	GAA	
V	I	K	M	K	A	L	I	F	A	A	A	G	L	L	L>	
	100			110			120				130			140		
*		*	*		*		*	*		*		*		*	*	
CTG	TTG	CCC	ACT	TTT	TGT	CAG	AGT	GGC	ATG	GAA	AAT	GAT	ACA	AAC	AAC	
GAC	AAC	GGG	TGA	AAA	ACA	GTC	TCA	CCG	TAC	CTT	TTA	CTA	TGT	TTG	TTG	
L	L	P	T	F	C	Q	S	G	M	E	N	D	T	N	N>	
	150			160			170				180			190		
*	*		*	*		*	*	*		*	*		*	*	*	
TTG	GCA	AAG	CCA	ACC	TTA	CCC	ATT	AAG	ACC	TTT	CGT	GGA	GCT	CCC	CCA	
AAC	CGT	TTC	GGT	TGG	AAT	GGG	TAA	TTC	TGG	AAA	GCA	CCT	CGA	GGG	GGT	
L	A	K	P	T	L	P	I	K	T	F	R	G	A	P	P>	
	200			210			220				230			240		
*	*		*	*		*	*	*		*	*		*	*	*	
AAT	TCT	TTT	GAA	GAG	TTC	CCC	TTT	TCT	GCC	TTG	GAA	GGC	TGG	ACA	GGA	
TTA	AGA	AAA	CTT	CTC	AAG	GGG	AAA	AGA	CGG	AAC	CTT	CCG	ACC	TGT	CCT	
N	S	F	E	E	F	P	F	S	A	L	E	G	W	T	G>	
	250			260			270				280					
*	*		*	*		*	*	*		*	*		*	*	*	
GCC	ACG	ATT	ACT	GTA	AAA	ATT	AAG	TGC	CCT	GAA	GAA	AGT	GCT	TCA	CAT	
CGG	TGC	TAA	TGA	CAT	TTT	TAA	TTC	ACG	GGA	CTT	CTT	TCA	CGA	AGT	GTA	
A	T	I	T	V	K	I	K	C	P	E	E	S	A	S	H>	
290			300				310				320			330		
*	*		*	*		*	*	*		*	*		*	*	*	
CTC	CAT	GTG	AAA	AAT	GCT	ACC	ATG	GGG	TAC	CTG	ACC	AGC	TCC	TTA	AGT	
GAG	GTA	CAC	TTT	TTA	CGA	TGG	TAC	CCC	ATG	GAC	TGG	TCG	AGG	AAT	TCA	
L	H	V	K	N	A	T	M	G	Y	L	T	S	S	L	S>	
	340			350			360				370			380		
*	*		*	*		*	*	*		*	*		*	*	*	
ACT	AAA	CTG	ATA	CCT	GCC	ATC	TAC	CTC	CTG	GTG	TTT	GTA	GTT	GGT	GTC	
TGA	TTT	GAC	TAT	GGA	CGG	TAG	ATG	GAG	GAC	CAC	AAA	CAT	CAA	CCA	CAG	
T	K	L	I	P	A	I	Y	L	L	V	F	V	V	G	V>	
	390			400			410				420			430		
*	*		*	*		*	*	*		*	*		*	*	*	
CCG	GCC	AAT	GCT	GTG	ACC	CTG	TGG	ATG	CTT	TTC	TTC	AGG	ACC	AGA	TCC	
GGC	CGG	TTA	CGA	CAC	TGG	GAC	ACC	TAC	GAA	AAG	AAG	TCC	TGG	TCT	AGG	
P	A	N	A	V	T	L	W	M	L	F	F	R	T	R	S>	



**Figure 1.** The effect of the number of iterations ( $n$ ) on the accuracy of the proposed algorithm. The figure shows two plots side-by-side. The left plot shows the error norm  $\|e\|_2$  versus  $n$ , ranging from 0 to 100. The right plot shows the relative error  $\frac{\|e\|_2}{\|x\|_2}$  versus  $n$ , ranging from 0 to 100. Both plots show a decreasing trend as  $n$  increases, indicating convergence.

		440			450			460			470			480		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
ATC	TGT	ACC	ACT	GTA	TTC	TAC	ACC	AAC	CTG	GCC	ATT	GCA	GAT	TTT	CTT	
TAG	ACA	TGG	TGA	CAT	AAG	ATG	TGG	TTG	GAC	CGG	TAA	CGT	CTA	AAA	GAA	
I	C	T	T	V	F	Y	T	N	L	A	I	A	D	F	L>	
		490			500			510			520					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
TTT	TGT	GTT	ACA	TTG	CCC	TTT	AAG	ATA	GCT	TAT	CAT	CTC	AAT	GGG	AAC	
AAA	ACA	CAA	TGT	AAC	GGG	AAA	TTC	TAT	CGA	ATA	GTA	GAG	TTA	CCC	TTG	
F	C	V	T	L	P	F	K	I	A	Y	H	L	N	G	N>	
530			540				550				560				570	
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
AAC	TGG	GTA	TTT	GGA	GAG	GTC	CTG	TGC	CGG	GCC	ACC	ACA	GTC	ATC	TTT	
TTG	ACC	CAT	AAA	CCT	CTC	CAG	GAC	ACG	GCC	CGG	TGG	TGT	CAG	TAG	AAG	
N	W	V	F	G	E	V	L	C	R	A	T	T	V	I	F>	
580			590				600				610				620	
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
TAT	GGC	AAC	ATG	TAC	TGC	TCC	ATT	CTG	CTC	CTT	GCC	TGC	ATC	AGC	ATC	
ATA	CCG	TTG	TAC	ATG	ACG	AGG	TAA	GAC	GAG	GAA	CGG	ACG	TAG	TCG	TAG	
Y	G	N	M	Y	C	S	I	L	L	L	A	C	I	S	I>	
630			640				650				660				670	
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
AAC	CGC	TAC	CTG	GCC	ATC	GTC	CAT	CCT	TTC	ACC	TAC	CGG	GGC	CTG	CCC	
TTG	GCG	ATG	GAC	CGG	TAG	CAG	GTA	GGA	AAG	TGG	ATG	GCC	CCG	GAC	GGG	
N	R	Y	L	A	I	V	H	P	F	T	Y	R	G	L	P>	
680			690				700				710				720	
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
AAG	CAC	ACC	TAT	GCC	TTG	GTA	ACA	TGT	GGA	CTG	GTG	TGG	GCA	ACA	GTT	
TTT	GTG	TGG	ATA	CGG	AAC	CAT	TGT	ACA	CCT	GAC	CAC	ACC	CGT	TGT	CAA	
K	H	T	Y	A	L	V	T	C	G	L	V	W	A	T	V>	
730			740				750				760					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
TTC	TTA	TAT	ATG	CTG	CCA	TTT	TTC	ATA	CTG	AAG	CAG	GAA	TAT	TAT	CTT	
AAG	AAT	ATA	TAC	GAC	GGT	AAA	AAG	TAT	GAC	TTC	GTC	CTT	ATA	ATA	GAA	
F	L	Y	M	L	P	F	F	I	L	K	Q	E	Y	Y	L>	
770			780				790				800				810	
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
GTT	CAG	CCA	GAC	ATC	ACC	ACC	TGC	CAT	GAT	GTT	CAC	AAC	ACT	TGC	GAG	
CAA	GTC	GGT	CTG	TAG	TGG	TGG	ACG	GTA	CTA	CAA	GTG	TTG	TGA	ACG	CTC	
V	Q	P	D	I	T	T	C	H	D	V	H	N	T	C	E>	
820			830				840				850				860	
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
TCC	TCA	TCT	CCC	TTC	CAA	CTC	TAT	TAC	TTC	ATC	TCC	TTG	GCA	TTC	TTT	
AGG	AGT	AGA	GGG	AAG	GTT	GAG	ATA	ATG	AAG	TAG	AGG	AAC	CGT	AAG	AAA	
S	S	S	P	F	Q	L	Y	Y	F	I	S	L	A	F	F>	

FIG. 3-3

```

      870      880      890      900      910
*      *      *      *      *      *      *      *      *
GGA TTC TTA ATT CCA TTT GTG CTT ATC ATC TAC TGC TAT GCA GCC ATC
CCT AAG AAT TAA GGT AAA CAC GAA TAG TAG ATG ACG ATA CGT CGG TAG
G   F   L   I   P   F   V   L   I   I   Y   C   Y   A   A   I>
      920      930      940      950      960
*      *      *      *      *      *      *      *      *
ATC CGG ACA CTT AAT GCA TAC GAT CAT AGA TGG TTG TGG TAT GTT AAG
TAG GCC TGT GAA TTA CGT ATG CTA GTA TCT ACC AAC ACC ATA CAA TTC
I   R   T   L   N   A   Y   D   H   R   W   L   W   Y   V   K>
      970      980      990      1000
*      *      *      *      *      *      *      *
GCG AGT CTC CTC ATC CTT GTG ATT TTT ACC ATT TGC TTT GCT CCA AGC
CGC TCA GAG GAG TAG GAA CAC TAA AAA TGG TAA ACG AAA CGA GGT TCG
A   S   L   L   I   L   V   I   F   T   I   C   F   A   P   S>
1010      1020      1030      1040      1050
*      *      *      *      *      *      *      *
AAT ATT ATT CTT ATT ATT CAC CAT GCT AAC TAC TAC TAC AAC AAC ACT
TTA TAA TAA GAA TAA TAA GTG GTA CGA TTG ATG ATG ATG TTG TTG TGA
N   I   I   L   I   I   H   H   A   N   Y   Y   Y   N   N   T>
1060      1070      1080      1090      1100
*      *      *      *      *      *      *      *
GAT GGC TTA TAT TTT ATA TAT CTC ATA GCT TTG TGC CTG GGT AGT CTT
CTA CCG AAT ATA AAA TAT ATA GAG TAT CGA AAC ACG GAC CCA TCA GAA
D   G   L   Y   F   I   Y   L   I   A   L   C   L   G   S   L>
1110      1120      1130      1140      1150
*      *      *      *      *      *      *      *
AAT AGT TGC TTA GAT CCA TTC CTT TAT TTT CTC ATG TCA AAA ACC AGA
TTA TCA ACG AAT CTA GGT AAG GAA ATA AAA GAG TAC AGT TTT TGG TCT
N   S   C   L   D   P   F   L   Y   F   L   M   S   K   T   R>
1160      1170      1180      1190      1200
*      *      *      *      *      *      *      *
AAT CAC TCC ACT GCT TAC CTT ACA AAA TAG TGA AAT GAT CTT AGA GAA
TTA GTG AGG TGA CGA ATG GAA TGT TTT ATC ACT TTA CTA GAA TCT CTT
N   H   S   T   A   Y   L   T   K   *   *   N   D   L   R   E>
      1210      1220
*      *      *      *
CAA GGA CAG CCA TCA CAG AGA ACG
GTT CCT GTC GGT AGT GTC TCT TGC
Q   G   Q   P   S   Q   R   T>

```

FIG. 4-1

10	20	30	40	50
* *	* *	* *	* *	* *
-ACAGGCATG	GAAAATGATA	CAAACAACTT	GGCAAAGCCA	ACCTTACCCA
60	70	80	90	100
* *	* *	* *	* *	* *
TTAAGACCTT	TCGTGGAGCT	CCCCCAAATT	CTTTTGAAGA	GTTCCCCTTT
110	120	130	140	150
* *	* *	* *	* *	* *
TCTGCCTTGG	AAGGCTGGAC	AGGAGCCACG	ATTACTGTAA	AAATTAAGTG
160	170	180	190	200
* *	* *	* *	* *	* *
CCCTGAAGAA	AGTGCTTCAC	ATCTCCATGT	GAAAAATGCT	ACCATGGGGT
210	220	230	240	250
* *	* *	* *	* *	* *
ACCTGACCAG	CTCCTTAAGT	ACTAAACTGA	TACCTGCCAT	CTACCTCCTG
260	270	280	290	300
* *	* *	* *	* *	* *
GTGTTTGTAG	TTGGTGTCCC	GGCCAATGCT	GTGACCCTGT	GGATGCTTTT
310	320	330	340	350
* *	* *	* *	* *	* *
CTTCAGGACC	AGATCCATCT	GTACCACTGT	ATTCTACACC	AACCTGGCCA
360	370	380	390	400
* *	* *	* *	* *	* *
TTGCAGATTT	TCTTTTTTGT	GTTACATTGC	CCTTTAAGAT	AGCTTATCAT
410	420	430	440	450
* *	* *	* *	* *	* *
CTCAATGGGA	ACAACTGGGT	ATTTGGAGAG	GTCCTGTGCC	GGGCCACCAC
460	470	480	490	500
* *	* *	* *	* *	* *
AGTCATCTTC	TATGGCAACA	TGTACTGCTC	CATTCTGCTC	CTTGCCTGCA
510	520	530	540	550
* *	* *	* *	* *	* *
TCAGCATCAA	CCGCTACCTG	GCCATCGTCC	ATCCTTTCAC	CTACCGGGGC
560	570	580	590	600
* *	* *	* *	* *	* *
CTGCCAAGC	ACACCTATGC	CTTGGTAACA	TGTGGACTGG	TGTGGGCAAC
610	620	630	640	650
* *	* *	* *	* *	* *
AGTTTTCTTA	TATATGCTGC	CATTTTTTCAT	ACTGAAGCAG	GAATATTATC

FIG. 4-2

660	670	680	690	700
* *	* *	* *	* *	* *
TTGTTTCAGCC	AGACATCACC	ACCTGCCATG	ATGTTCACAA	CACTTGCGAG
710	720	730	740	750
* *	* *	* *	* *	* *
TCCTCATCTC	CCTTCCAAC	CTATTACTTC	ATCTCCTTGG	CATTCTTTGG
760	770	780	790	800
* *	* *	* *	* *	* *
ATTCTTAATT	CCATTTGTGC	TTATCATCTA	CTGCTATGCA	GCCATCATCC
810	820	830	840	850
* *	* *	* *	* *	* *
GGACACTTAA	TGCATACGAT	CATAGATGGT	TGTGGTATGT	TAAGGCGAGT
860	870	880	890	900
* *	* *	* *	* *	* *
CTCCTCATCC	TTGTGATTTT	TACCATTTGC	TTTGCTCCAA	GCAATATTAT
910	920	930	940	950
* *	* *	* *	* *	* *
TCTTATTATT	CACCATGCTA	ACTACTACTA	CAACAACACT	GATGGCTTAT
960	970	980	990	1000
* *	* *	* *	* *	* *
ATTTTATATA	TCTCATAGCT	TTGTGCCTGG	GTAGTCTTAA	TAGTTGCTTA
1010	1020	1030	1040	1050
* *	* *	* *	* *	* *
GATCCATTCC	TTTATTTTCT	CATGTCAAAA	ACCAGAAATC	ACTCCACTGC
1060	1070	1080	1090	1100
* *	* *	* *	* *	* *
TTACCTTACA	AAATAGTGAA	ATGATCTTAG	AGAACAAGGA	CAGCCATCAC

AGA



FIG. 6 is a bar graph showing the surface staining of M1-epitope +/-  $\alpha$ -thrombin at 37°C for 5 minutes. The y-axis represents the staining intensity from 0 to 1. The x-axis shows four groups of transfectants: hPAR1, hPAR3, hPAR3 T39P, and TRANSFECTANTS. For each group, two bars are shown: 'no' (white) and ' $\alpha$ Th 20nM' (stippled). Error bars are present for all bars.

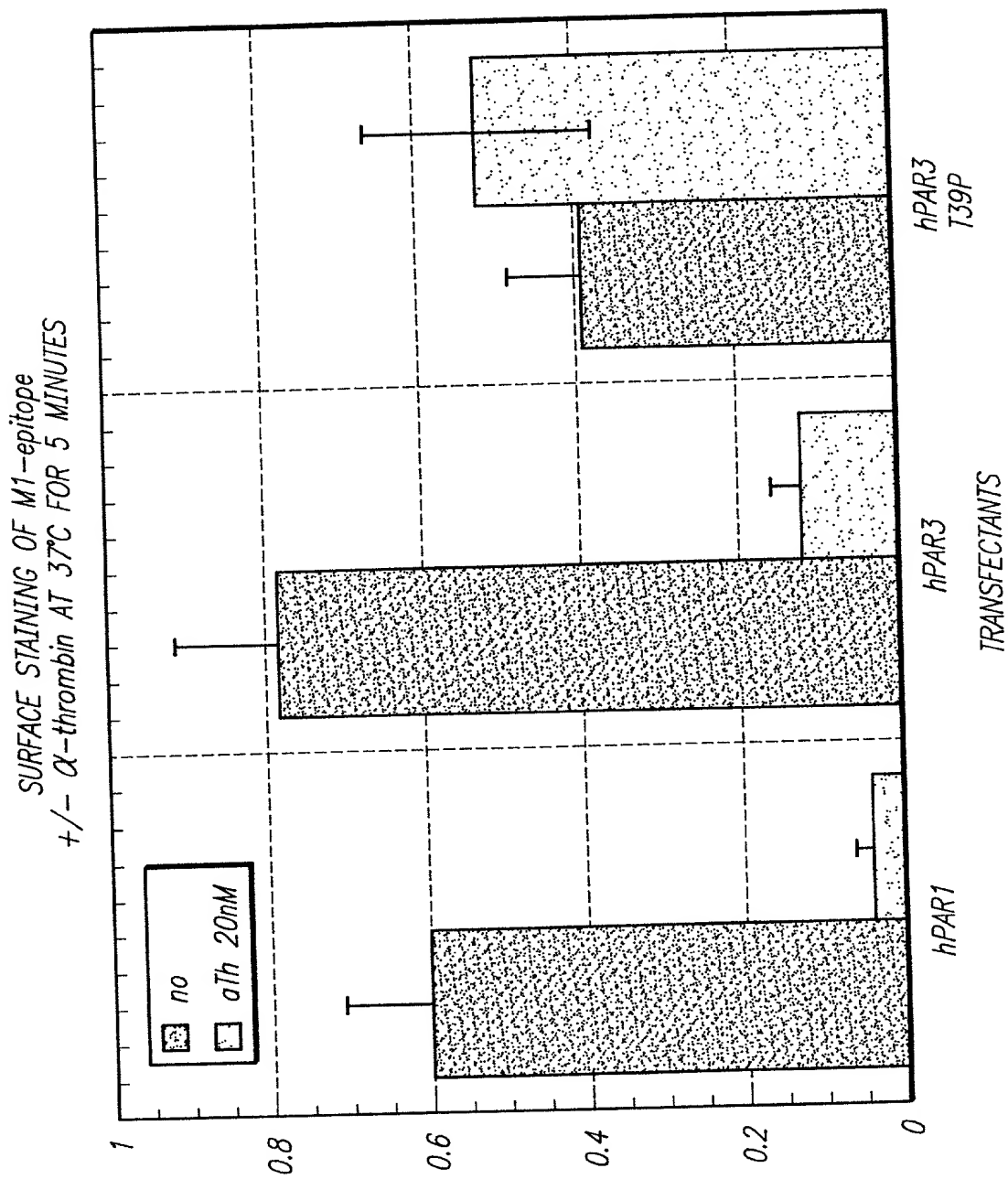


FIG. 6

FIG. 7

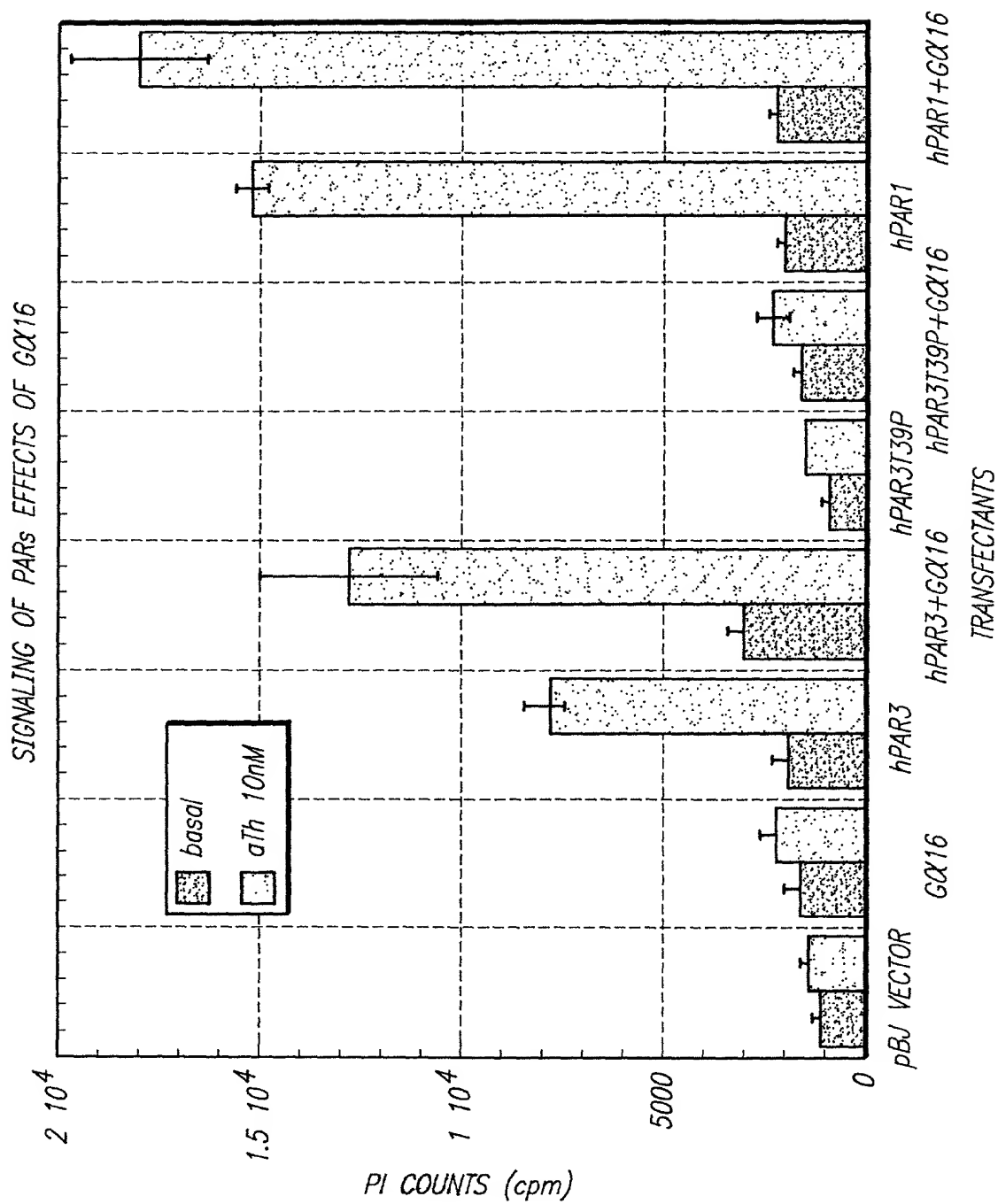


FIG. 8

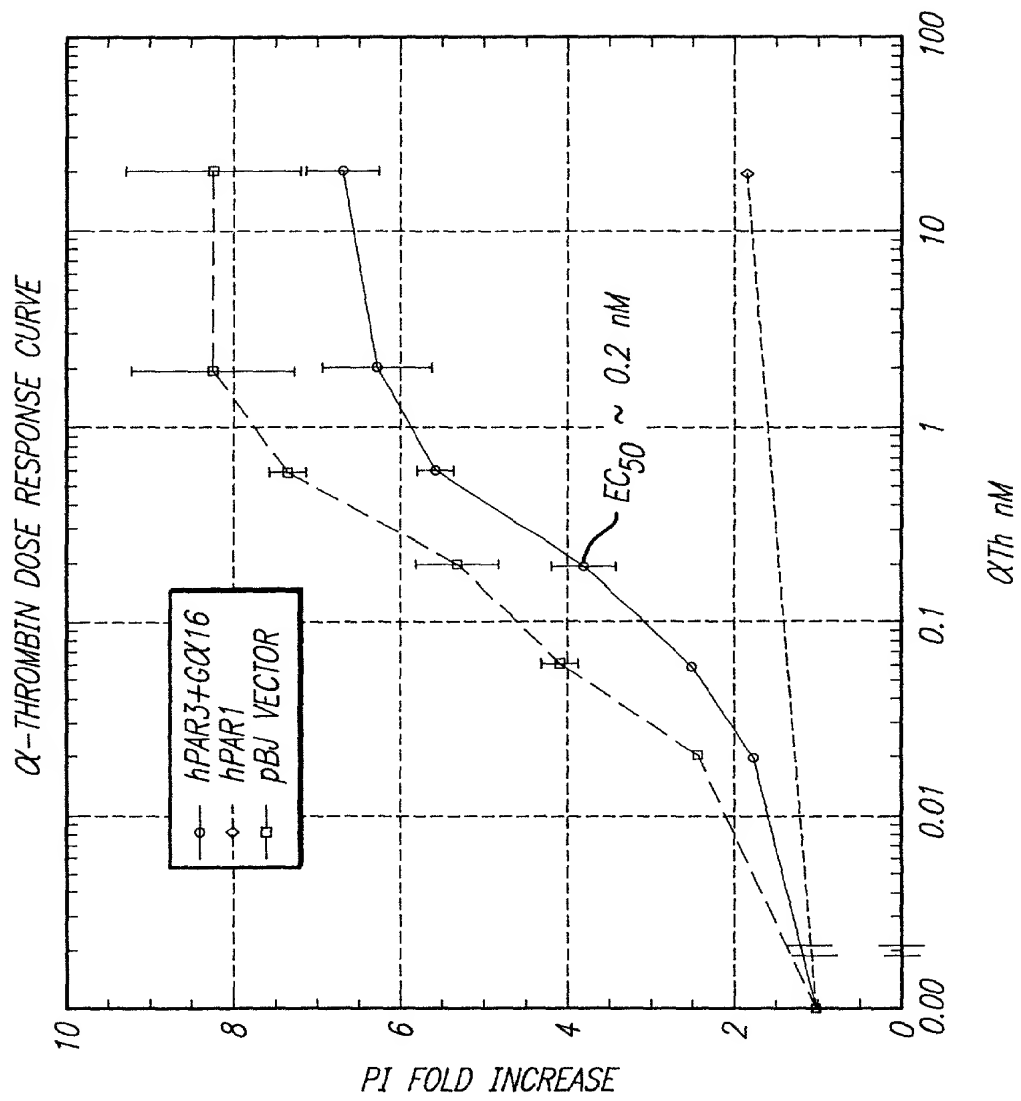
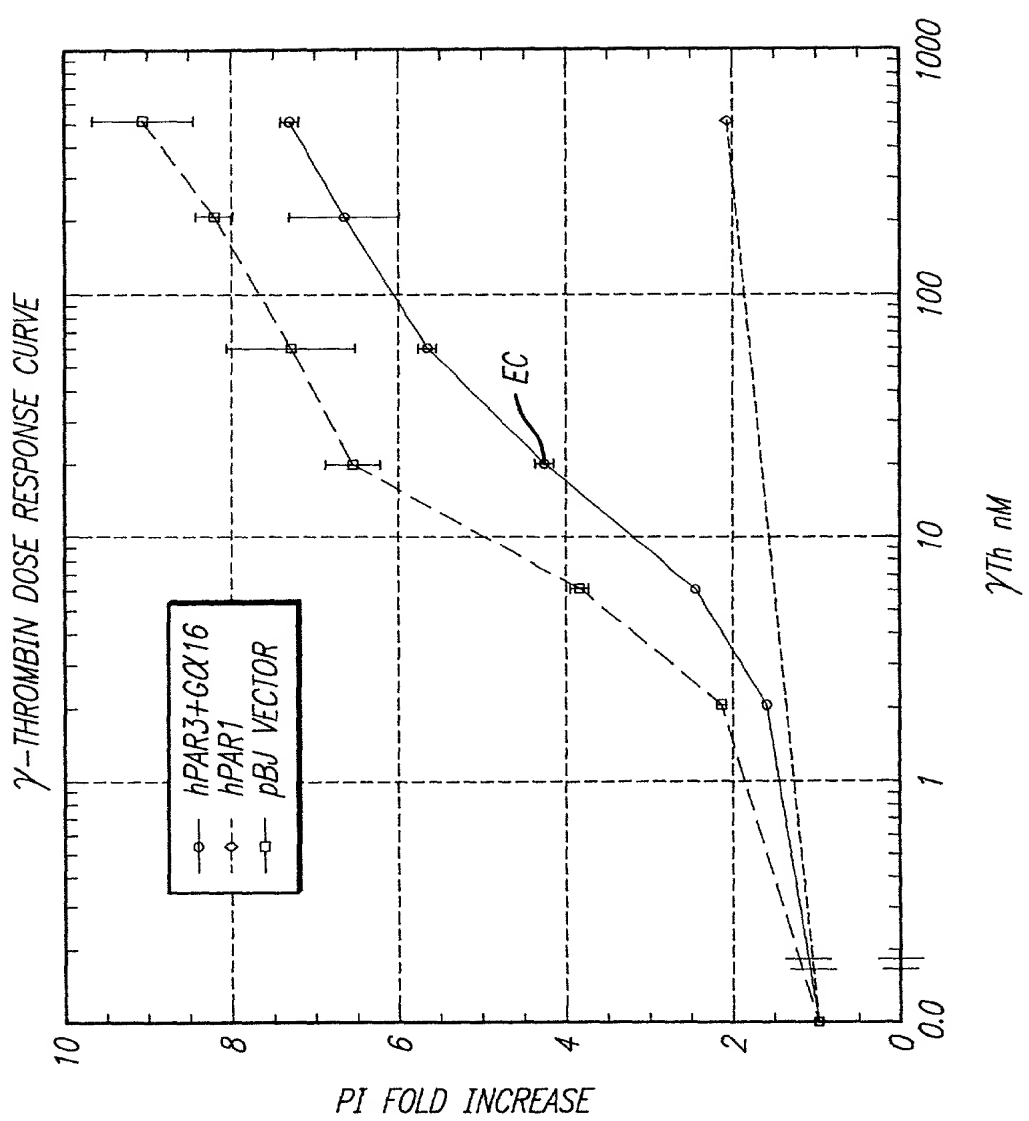




FIG. 9

FIG. 9



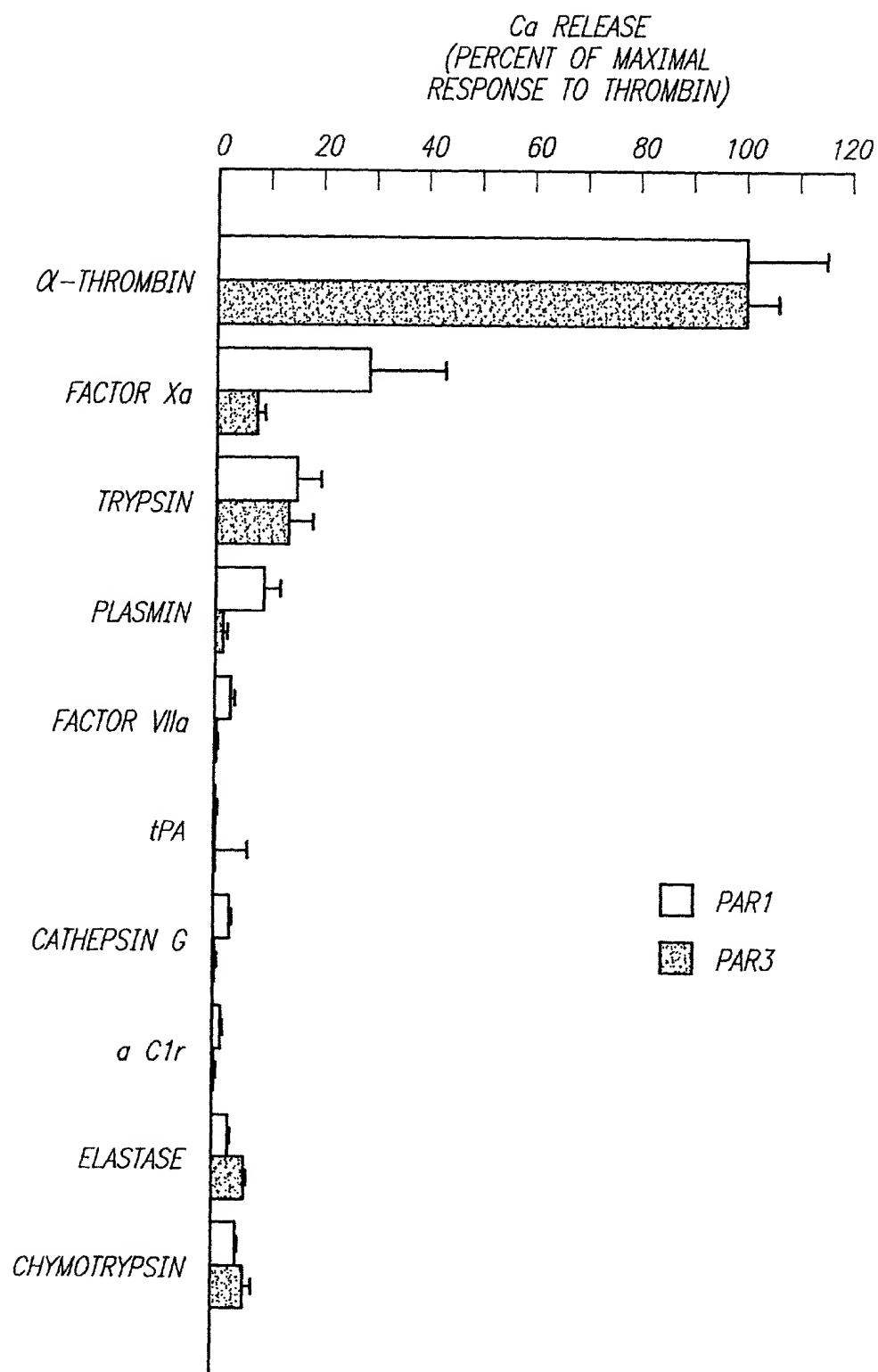


FIG. 10

10 20 30 40 50  
 TG ACT TTG TAT ACT TAA CAA CAT CCT GTA GCC OGG TCT CAG GAC ATC AAG  
 AC TGA AAC ATA TGA ATT GTT GTA OGA CAT CCG CCC AGA GTC CTG TAG TTC  
 T L Y T Q H P V A G S Q D I K>

60 70 80 90  
 ATG AAA ATC CTT ATC TTG GTT GCA GCT OGG CTG CTG TTT CTG CCA GTC  
 TAC TTT TAG GAA TAG AAC CAA COT CGA CCC GAC GAC AAA GAC GGT CAG  
 M K I L I L V A A G L L F L P V>

100 110 120 130 140  
 ACT GTT TGC CAA AGT GGC ATA AAT GTT TCA GAC AAC TCA GCA AAG CCA  
 TGA CAA ACG GTT TCA CCG TAT TTA CAA AGT CTG TTG AGT COT TTC GGT  
 T V C Q S G I N V S D N S A R P>

150 160 170 180 190  
 ACC TTA ACT ATT AAG AGT TTT AAT OGG GGT CCC CAA AAT ACC TTT GAA  
 TGG AAT TGA TAA TTC TCA AAA TTA CCC CCA OGG GTT TTA TGG AAA CTT  
 T L T I R S F N G G P Q N T F E>

200 210 220 230 240  
 GAA TTC CCA CTT TCT GAC ATA GAG OGC TGG ACA GGA GGC ACC ACA ACT  
 CTT AAG GGT GAA AGA CTG TAT CTC CCG ACC TGT CCT CGG TGG TGT TGA  
 E F P L S D I E G W T G A T T T>

250 260 270 280 290  
 ATA AAA OGC GAG TOT CCC GAG GAC AGT ATT TCA ACT CTC CAC GTG AAT  
 TAT TTT CGC CTC ACA GGG CTC CTG TCA TAA AGT TGA GAG GTG CAC TTA  
 I K A E C P E D S I S T L H V N>

300 310 320 330  
 AAT GCT ACC ATA GGA TAC CTG AGA AGT TCC TTA AGT ACC CAA GTG ATA  
 TTA CGA TGG TAT CCT ATG GAC TCT TCA AGG AAT TCA TGG GTT CAC TAT  
 N A T I G Y L R S S L S T Q V I>

340 350 360 370 380  
 CCT GGC ATC TAT ATC CTG CTG TTT GTG GTT GGT GTA CCA TCC AAC ATC  
 GGA CGG TAG ATA TAG GAC GAC AAA CAC CAA CCA CAT GGT AGG TTG TAG  
 P A I Y I L L F V V G V P S N I>

390 400 410 420 430  
 GTG ACC CTG TGG AAA CTC TCC TTA AGG ACC AAA TCC ATC AGT CTG GTC  
 CAC TGG GAC ACC TTT GAG AGG AAT TCC TGG TTT AGG TAG TCA GAC CAG  
 V T L W R L S L R T K S I S L V>

440 450 460 470 480  
 ATC TTT CAC ACC AAC CTG GCC ATC GCA GAT CTC CTT TTC TOT GTC ACA  
 TAG AAA GTG TGG TTG GAC CGG TAG COT CTA GAG GAA AAG ACA CAG TOT  
 I F H T M L A I A D L L F C V T>

FIG. 1 1 of 3

490 500 510 520 530  
 CTG CCA TTT AAG ATC GCC TAC CAT CTC AAT GGC AAC AAC TCG GTA TTT  
 GAC GGT AAA TTC TAG CGG ATG GTA GAG TTA CCG TTG TTG ACC CAT AAA  
 L P P K I A Y H L N G N N W V F>  
 540 550 560 570  
 GGC GAG GTC ATG TGC CGG ATC ACC ACG GTC GTT TTC TAC GGC AAC ATG  
 CCG CTC CAG TAC ACG GCC TAG TGG TGC CAG CAA AAG ATG CCG TTG TAC  
 G E V M C R I T T V V F Y G N M>  
 580 590 600 610 620  
 TAC TGC GCT ATC CTG ATC CTC ACT TGC ATG GGC ATC AAC CGC TAC CTG  
 ATG ACG CGA TAG GAC TAG GAG TGA ACG TAC CCG TAG TTG GCG ATG GAC  
 Y C A I L I L T C M G I N R Y L>  
 630 640 650 660 670  
 GCC ACG GCT CAC CCT TTC ACA TAC CAG AAG CTG CCC AAA CGC AGC TTC  
 CCG TGC CGA GTG GGA AAG TGT ATG GTC TTC GAC GCG TTT GCG TCG AAG  
 A T A H P F T Y Q K L P K R S F>  
 680 690 700 710 720  
 TCC TTG CTC ATG TGT GGC ATA GTG TGG GTC ATG GTT TTC TTA TAC ATG  
 AGG AAC GAG TAC ACA CCG TAT CAC ACC CAG TAC CAA AAG AAT ATG TAC  
 S L L M C G I V W V H V F L Y M>  
 730 740 750 760 770  
 CTG CCC TTT GTC ATC CTG AAG CAG GAG TAC CAC CTC GTC CAC TCA GAG  
 GAC GGG AAA CAG TAG GAC TTC GTC CTC ATG GTG GAG CAG GTG AGT CTC  
 L P P V I L K Q E Y H L V H S E>  
 780 790 800 810  
 ATC ACC ACC TGC CAC GAT GTC GTC GAC GCG TGC GAG TCC CCA TCA TCC  
 TAG TGG TGG ACG GTG CTA CAG CAG CTG CCG ACG CTC ACG GGT AGT AGG  
 I T T C H D V V D A C E S P S S>  
 820 830 840 850 860  
 TTC CGA TTC TAC TAC TTC GTC TCC TTA GCA TTC TTT GGG TTC CTC ATC  
 AAG GCT AAG ATG ATG AAG CAG AAG AAT CGT AAG AAA CCC AAG GAG TAG  
 P R P Y Y F V S L A P P G F L I>  
 870 880 890 900 910  
 CCG TTT GTG ATC ATC ATC TTC TGT TAC ACG ACT CTC ATC CAC AAA CTT  
 GGC AAA CAC TAG TAG TAG AAG ACA ATG TGC TGA GAG TAG GTG TTT GAA  
 P P V I I I F C Y T T L I H K L>  
 920 930 940 950 960  
 AAA TCA AAG GAT CCG ATA TGG CTG GGC TAC ATC AAG GGC GTC CTC CTC  
 TTT AOT TTC CTA GCC TAT ACC GAC CCG ATG TAG TTC CCG CAG GAG GAG  
 K S K D R I W L G Y I K A V L L>  
 970 980 990 1000 1010

FIG. 1 2 of 3

ATC GTT GTG ATT TTC ACA ATT TGC TTT GCC CCC ACC AAC ATC ATA CTC  
TAG GAA CAC TAA AAG TGT TAA ACG AAA CCG GCG TCG TTG TAG TAT GAG  
I L V I F T I C F A P T N I I L>

1020 1030 1040 1050  
GTA ATC CAC CAT GCC AAC TAC TAC TAC CAC AAT ACC GAC AGC TTG TAC  
CAT TAG GTG GTA CCG TTG ATG ATG ATG GTG TTA TCG CTG TCG AAC ATG  
V I H H A N Y Y Y H N T D S L Y>

1060 1070 1080 1090 1100  
TTT ATG TAT CTT ATT GCT CTG TGC CTG GGG AGC CTG AAT AGC TGC CTA  
AAA TAC ATA GAA TAA CGA GAC ACG GAC CCC TCG GAC TTA TCG ACG GAT  
F H Y L I A L C L G S L N S C L>

1110 1120 1130 1140 1150  
GAT CCA TTC CTT TAC TTT GTC ATG TCG AAA GTT GTA GAT CAG CTT AAT  
CTA GGT AAG GAA ATG AAA CAG TAC AGC TTT CAA CAT CTA GTC GAA TTA  
D P F L Y F V M S K V V D Q L N>

1160 1170 1180 1190 1200  
CCT TAG TCG GCA ATG GCA AGA CCA CTT TAG AGA CCA AGG AGA GAT ATC  
GGA ATC AGC CGT TAC CGT TCT GGT GAA ATC TCT GGT TCC TCT CTA TAG  
P \* S A M A R P L \* R P R R D I>

1210 1220  
TGG GAA GAC ATA CAT GCT TGG C  
ACC CTT CTG TAT GTA CGA ACC G  
W E D I H A W X>

FIG. 1 3 of 3

10 20 30 40 50  
 . . . . .  
 CCATATGCTA ATATTTCCTT TCAATTACAG GCATAAATGT TTCAGACAAC  
 60 70 80 90 100  
 . . . . .  
 TCAGCAAAGC CAACCTTAAC TATTAAGAGT TTTAATGGGG GTCCCAAAA  
 110 120 130 140 150  
 . . . . .  
 TACCTTTGAA GAATTC-----TACAACCT CTCCATGTGA ATAATGCTAC  
 160 170 180 190 200  
 . . . . .  
 CATGGGATAC CTGAGAAGTT CCTTAAGTAC CAAAGTGATA CCTGCCATCT  
 210 220 230 240 250  
 . . . . .  
 ACATCCTGGT GTTTGTGATT GGTGTACCAG CGAACATCGT GACCCGTGG  
 260 270 280 290 300  
 . . . . .  
 AAACCTCTCT CAAGGACCAA ATCCATCTGT CTGGTCACTT TTCACACCAA  
 310 320 330 340 350  
 . . . . .  
 CCTGGCCATC GCGGATCTCC TTTTCTGTGT CACGCTGCCG TTAAAGATC-  
 360 370 380 390 400  
 . . . . .  
 -CCTACCATC TCAATGGCAA CAACTGGGTA TTGGCGAAG TCATGTGCCG  
 410 420 430 440 450  
 . . . . .  
 GATCACCACG GTCGTTTTCT ACGGCAACAT GTACTGCCCT A---TCCTGA  
 460 470 480 490 500  
 . . . . .  
 TCCTCACCTG CATGGGCATC AACCGCTACC TGGCCACGGC TCACCCTTTC  
 510 520 530 540 550  
 . . . . .  
 ACATACCAGA AGCTGCCCCA ACGCAGCTTC TCCATGCTCA TGTGTGGCAT  
 560 570 580 590 600  
 . . . . .  
 GGTGTGGGTC ATGGTTTTCT TATACATGCT GCCCTTTGTC ATCC---AAG  
 610 620 630 640 650  
 . . . . .  
 CAGGAGTACC ACCTCGTCCA CTCGGAGATC ACCACCTGCC ACGATGTCGT  
 660 670 680 690 700  
 . . . . .  
 CGACGGGTGC GANTCCCCAT CATCCTTCCG ATTCTACTAC TTGGTCTCTT  
 710 720 730 740 750

FIG. 2 1 of 2

. . . . .  
 TAGCATTCTT TGGGTTCTCT ATCCCGTTTG TGATCATCAT CTTCTGTTAC  
 760 770 780 790 800  
 . . . . .  
 ACGACTCTCA TCCACAAACT TAAATCAAAA GATCNGATAT GGCTGGGCTA  
 810 820 830 840 850  
 . . . . .  
 CATCAAGGCC GTCTCTCTCA TCCTTG TGAA TTTCACCATC TGCTTCCCCC  
 860 870 880 890 900  
 . . . . .  
 CCACCAAG-- ----GATATC TGGGAAGACG TACATGCTTG GCTGACTTGT  
 910 920 930 940 950  
 . . . . .  
 GCATGGCACC ATCAGCTCAA TTTTAAATTT TTTAATTTTA ATTTAATTTA  
 960 970 980 990 1000  
 . . . . .  
 ATTTTATGTT TTTGAGACAG AGCCTCACTG TGTAGTCCTG GCTGGCCTGG  
 1010 1020 1030 1040 1050  
 . . . . .  
 CTGGTTCTCT ATTTAGACCA GGTTAGCCTT GAACTCACAG AGATCTGCCT  
 1060 1070 1080 1090 1100  
 . . . . .  
 GCTTCTGCCT CCCAAGTGCT GGGTCAACC AGGTCTGGCA AGCGCTCCAT  
 1110 1120  
 . . . . .  
 TTTTCAGCTC CTCTGCAACA GTGC

**FIG. 2 2 of 2**

10 20 30 40  
 TGC TCC ATG ATT TTA CAG ATT TCA TAA CGT TTA AGA GAC GGG ACT CAG  
 ACC AGG TAC TAA AAT GTC TAA AGT ATT GCA AAT TCT CTG CCC TGA GTC  
 C S M I L Q I S \* R L R D G T Q>  
 50 60 70 80 90  
 GTC ATC AAA ATG AAA GCC CTC ATC TTT GCA GCT GCT GGC CTC CTG CTT  
 CAG TAG TTT TAC TTT CGG GAG TAG AAA CGT CGA CGA CCG GAG GAC GAA  
 V I K M K A L I F A A A G L L L>  
 100 110 120 130 140  
 CTG TTG CCC ACT TTT TGT CAG AGT GGC ATG GAA AAT GAT ACA AAC AAC  
 GAC AAC GGG TGA AAA ACA GTC TCA CCG TAC CTT TTA CTA TGT TTG TTG  
 L L P T F C Q S G M E N D T N N>  
 150 160 170 180 190  
 TTG GCA AAG CCA ACC TTA CCC ATT AAG ACC TTT CGT GGA GCT CCC CCA  
 AAC CGT TTC GGT TGG AAT GGG TAA TTC TGG AAA GCA CCT CGA GGG GGT  
 L A K P T L P I K T F R G A P P>  
 200 210 220 230 240  
 AAT TCT TTT GAA GAG TTC CCC TTT TCT GCC TTG GAA GGC TGG ACA GGA  
 TTA AGA AAA CTT CTC AAG GGG AAA AGA CGG AAC CTT CCG ACC TGT CCT  
 N S F E E F P F S A L E G W T G>  
 250 260 270 280  
 GCC ACG ATT ACT GTA AAA ATT AAG TGC CCT GAA GAA AGT GCT TCA CAT  
 CGG TGC TAA TGA CAT TTT TAA TTC ACG GGA CTT CTT TCA CGA AGT GTA  
 A T I T V K I K C P E E S A S H>  
 290 300 310 320 330  
 CTC CAT GTG AAA AAT GCT ACC ATG GGG TAC CTG ACC AGC TCC TTA AGT  
 GAG GTA CAC TTT TTA CGA TGG TAC CCC ATG GAC TGG TCG AGG AAT TCA  
 L H V K N A T M G Y L T S S L S>  
 340 350 360 370 380  
 ACT AAA CTG ATA CCT GCC ATC TAC CTC CTG GTG TTT GTA GTT GGT GTC  
 TGA TTT GAC TAT CCA GCG TAG ATG GAG GAC CAC AAA CAT CAA CCA CAG  
 T K L I P A I Y L L V F V V G V>  
 390 400 410 420 430  
 CCG GCC AAT GCT GTG ACC CTG TGG ATG CTT TTC TTC AGG ACC AGA TCC  
 GGC CGG TTA CGA CAC TGG GAC ACC TAC GAA AAG AAG TCC TGG TCT AGG  
 P A N A V T L W M L F F R T R S>  
 440 450 460 470 480  
 ATC TGT ACC ACT GTA TTC TAC ACC AAC CTG GCC ATT GCA GAT TTT CTT  
 TAG ACA TGG TGA CAT AAG ATG TGG TTG GAC CGG TAA CGT CTA AAA GAA  
 I C T T V F Y T N L A I A D F L>

FIG. 3 1 of 3



490 500 510 520  
 TTT TGT GTT ACA TTG CCC TTT AAG ATA GCT TAT CAT CTC AAT GGG AAC  
 AAA ACA CAA TGT AAC GGG AAA TTC TAT CGA ATA GTA GAG TTA CCC TTG  
 F C V T L P F K I A Y H L N G N>  
 530 540 550 560 570  
 AAC TGG GTA TTT GGA GAG GTC CTG TGC CGG GCC ACC ACA GTC ATC TTC  
 TTG ACC CAT AAA CCT CTC CAG GAC ACG GCC CGG TGG TGT CAG TAG AAG  
 N W V F G E V L C R A T T V I F>  
 580 590 600 610 620  
 TAT GGC AAC ATG TAC TGC TCC ATT CTG CTC CTT GCC TGC ATC AGC ATC  
 ATA CCG TTG TAC ATG ACG AGG TAA GAC GAG GAA CGG ACG TAG TCG TAG  
 Y G N M Y C S I L L L A C I S I>  
 630 640 650 660 670  
 AAC CGC TAC CTG GCC ATC GTC CAT CCT TTC ACC TAC CGG GGC CTG CCC  
 TTG GCG ATG GAC CGG TAG CAG GTA GGA AAG TGG ATG GCC CCG GAC GGG  
 N R Y L A I V H P F T Y R G L P>  
 680 690 700 710 720  
 AAG CAC ACC TAT GCC TTG GTA ACA TGT GGA CTG GTG TGG GCA ACA GTT  
 TTC GTG TGG ATA CCG AAC CAT TGT ACA CCT GAC CAC ACC CGT TGT CAA  
 K H T Y A L V T C G L V W A T V>  
 730 740 750 760  
 TTC TTA TAT ATG CTG CCA TTT TTC ATA CTG AAG CAG GAA TAT TAT CTT  
 AAG AAT ATA TAC GAC GGT AAA AAG TAT GAC TTC GTC CTT ATA ATA GAA  
 F L Y M L P F F I L K Q E Y Y L>  
 770 780 790 800 810  
 GTT CAG CCA GAC ATC ACC ACC TGC CAT GAT GTT CAC AAC ACT TGC GAG  
 CAA GTC GGT CTG TAG TGG TGG ACG GTA CTA CAA GTG TTG TGA ACG CTC  
 V Q P D I T T C H D V H N T C E>  
 820 830 840 850 860  
 TCC TCA TCT CCC TTC CAA CTC TAT TAC TTC ATC TCC TTG GCA TTC TTT  
 ACG AGT AGA GGG AAG GTT GAG ATA ATG AAG TAG AGG AAC CGT AAG AAA  
 S S S P F Q L Y Y F I S L A F P>  
 870 880 890 900 910  
 GGA TTC TTA ATT CCA TTT GTG CTT ATC ATC TAC TGC TAT GCA GCC ATC  
 CCT AAG AAT TAA GGT AAA CAC GAA TAG TAG ATG ACG ATA CGT CGG TAG  
 G F L I P F V L I I Y C Y A A I>  
 920 930 940 950 960  
 ATC CGG ACA CTT AAT GCA TAC GAT CAT AGA TGG TTG TGG TAT GTT AAG  
 TAG GCC TGT GAA TTA CGT ATG CTA GTA TCT ACC AAC ACC ATA CAA TTC  
 I R T L N A Y D H R W L W Y V K>  
 970 980 990 1000

FIG. 3 2 of 3

CCG AGT CTC CTC ATC CTT GTG ATT TTT ACC ATT TGC TTT GCT CCA AGC  
 CGC TCA GAG GAG TAG GAA CAC TAA AAA TCG TAA ACG AAA CGA GGT TCG  
 A S L L I L V I F T I C F A P S>

1010 1020 1030 1040 1050  
 AAT ATT ATT CTT ATT ATT CAC CAT GCT AAC TAC TAC TAC AAC AAC ACT  
 TTA TAA TAA GAA TAA TAA GTG GTA CGA TTT ATG ATG ATG TTG TTG TGA  
 N I I L I I H H A N Y Y Y N N T>

1060 1070 1080 1090 1100  
 GAT GGC TTA TAT TTT ATA TAT CTC ATA GCT TTG TGC CTG GGT AGT CTT  
 CTA CCG AAT ATA AAA TAT ATA GAG TAT CGA AAC ACG GAC CCA TCA GAA  
 D G L Y F I Y L I A L C L G S L>

1110 1120 1130 1140 1150  
 AAT AGT TGC TTA GAT CCA TTC CTT TAT TTT CTC ATG TCA AAA ACC AGA  
 TTA TCA ACG AAT CTA GGT AAG GAA ATA AAA GAG TAC AGT TTT TGG TCT  
 N S C L D P F L Y F L M S K T R>

1160 1170 1180 1190 1200  
 AAT CAC TCC ACT GCT TAC CTT ACA AAA TAG TGA AAT GAT CTT AGA GAA  
 TTA GTG AGG TGA CGA ATG GAA TGT TTT ATC ACT TTA CTA GAA TCT CTT  
 N H S T A Y L T K \* \* N D L R E>

1210 1220  
 CAA GGA CAG CCA TCA CAG AGA ACG  
 GTT CCT GTC GGT AGT GTC TCT TGC  
 Q G Q P S Q R T>

FIG. 3 3 of 3

```

      10      20      30      40      50
      .      .      .      .      .
-ACAGGCATG GAAAATGATA CAAACAACCTT GGCAAAGCCA ACCTTACCCA

      60      70      80      90     100
      .      .      .      .      .
TTAAGACCTT TCGTGGAGCT CCCCCAAATT CTTTGAAGA GTTCCCCTTT

      110     120     130     140     150
      .      .      .      .      .
TCTGCCTTGG AAGGCTGGAC AGGAGCCACG ATTACTGTAA AAATTAAGTG

      160     170     180     190     200
      .      .      .      .      .
CCCTGAAGAA AGTGCTTCAC ATCTCCATGT GAAAAATGCT ACCATGGGGT

      210     220     230     240     250
      .      .      .      .      .
ACCTGACCAG CTCCTTAAGT ACTAAACTGA TACCTGCCAT CTACCTCCTG

      260     270     280     290     300
      .      .      .      .      .
GTGTTTGTAG TTGGTGTCCC GGCCAATGCT GTGACCCTGT GGATGCTTTT

      310     320     330     340     350
      .      .      .      .      .
CTTCAGGACC AGATCCATCT GTACCACTGT ATTCTACACC AACCTGGCCA

      360     370     380     390     400
      .      .      .      .      .
TTGCAGATTT TCTTTTTTGT GTTACATTGC CCTTTAAGAT AGCTTATCAT

      410     420     430     440     450
      .      .      .      .      .
CTCAATGGGA ACAACTGGGT ATTTGGAGAG GTCCTGTGCC GGGCCACCAC

      460     470     480     490     500
      .      .      .      .      .
AGTCATCTTC TATGGCAACA TGTACTGCTC CATTCGTCTC CTTGCCTGCA

      510     520     530     540     550
      .      .      .      .      .
TCAGCATCAA CCGCTACCTG GCCATCGTCC ATCCTTTCAC CTACCGGGGC

      560     570     580     590     600
      .      .      .      .      .
CTGCCCAAGC ACACCTATGC CTTGGTAACA TGTGGACTGG TGTGGGCAAC

      610     620     630     640     650
      .      .      .      .      .
AGTTTTCTTA TATATGCTGC CATTTTCAT ACTGAAGCAG GAATATTATC

      660     670     680     690     700
      .      .      .      .      .
TTGTTTCAGC AGACATCACC ACCTGCCATG ATGTTTACAA CACTTGGGAG

      710     720     730     740     750

```

FIG. 4 1 of 2

TCCTCATCTC CCTTCCAAC TATTACTTC ATCTCCTTGG CATTCTTTGG  
 760 770 780 790 800  
 ATTCTTAATT CCATTGTGTC TTATCATCTA CTGCTATGCA GCCATCATCC  
 810 820 830 840 850  
 GGACACTTAA TGCATACGAT CATAGATGGT TGTGGTATGT TAAGGCGAGT  
 860 870 880 890 900  
 CTCCTCATCC TTGTGATTTT TACCATTGTC TTTGCTCCAA GCAATATTAT  
 910 920 930 940 950  
 TCTTATTATT CACCATGCTA ACTACTACTA CAACAACACT GATGGCTTAT  
 960 970 980 990 1000  
 ATTTTATATA TCTCATAGCT TTGTGCCTGG GTAGTCTTAA TAGTTGCTTA  
 1010 1020 1030 1040 1050  
 GATCCATTCC TTTATTTTCT CATGTCAAAA ACCAGAAATC ACTCCACTGC  
 1060 1070 1080 1090 1100  
 TTACCTTACA AAATAGTGAA ATGATCTTAG AGAACAAGGA CAGCCATCAC  
 AGA

**FIG. 4 2 of 2**

a. AMINO ACID SEQUENCE OF PROTEASE-ACTIVATED RECEPTOR 3: COMPARISON WITH PAR1 AND PAR2.

hPAR3- 1 MKA LIFAAAGLLLLLP TFGSGKHNHNNLAKP TLPIK/TERGAPPN SFEFFPFSALEGWTGATITKIK PEESASHLVYKNAQMG  
hPAR1- 1 MGPRR LLLVAACFSUCDF LLSAATNARAPESKATNLTDFR SFLLRNPNCKYEPFWEDEEKNESQLTETPLVSIHYSSPLQKQLPAFISECASC  
hPAR2- 1 MRSPSAAWLLGAATLLA ASLSGDTIQG TNRSSKGR SLIGKVDGTSHTVTKGVTV ETVFSVDEFSAS

hPAR3- 87 YLTSSLSSTKLIPAIYLLVYVGVPAHAVTLMNLFRT SICTTATYTNLAIAOFLFCVTLPIKIAHUNNNMVFGEVLCRATTVIFYGNMYCSILLACISINRYLA  
hPAR1- 95 YLTSSWLTFLVPSVYTGVPVSLPNIMAIWVFILKMKVKKPAVVMKMLATADVLSVLPFKISYFSGSDWQFSGELCRFVTAIFYCNMYASILLNTVISTDRFLAV  
hPAR2- 68 VLTGKLTTFELP:VYTVIFVVGCPNSGMALWVLFRTKKKHFAVIMANLALADLLSVIWFPLFIAYHINQNMIFYGALCNVLIGFFYGNMYCSILENTCLSVQRYWV

hPAR3- 136 VHPFTYRGLPKHTYALVTGGLWATVFLYMLPFFILKQEYVLVQPDITTHOVHNTGESSSPFQLYYFISLAFPGFLIPFVL:YCYAAIIRTNA YCHRWLAAY  
hPAR1- 205 VYPMQSLSWRTGSRASFTCLAIWALAIAGVPLVVKQETIQVPGLNITTHOVUNETLLEQ YYAYYFSAFSAVFFVPLIISTVCYVSI:RCLSSSAVANRSKK SPAL  
hPAR2- 178 VNPNGHSRRKANALIS: SLAIWLLILLVTIPLVYVQTIIFIPALNITTHOVLPQELVGO KPNYFLSLAIGVFLPAFLTASAYVLMIRMLRSSAHENSEKKRKA

hPAR3- 301 KASLLILVIFTICFAPSNIILIKHANYYYNNT OGlyFIYLIALLGSLNSCLDPFLYFLMSKTRNMSTAYLTK  
hPAR1- 313 FLAAVFCIFILICFQPTNVLIIAHYSFLSHTSTTEAAYFAYLLGVYSSISSCIDPLIYYASSECQRYVYSILCKESSDPSSYNSSGQLMASHKMDTSSNLMNSIYKLL  
hPAR2- 287 KLIVTVLAMYLICFTPSNLLLVVHY FLIKSQGSHVYALYIVALCLSTLNSCIDPFVYVYFVSHDFRMAKNALLCRSVRTVKQMVSLSKXHSRKSSSYSSSSTTVKTS

b. FEATURES OF PAR AMINO TERMINAL EXODOMAINS.

Hirudin C-tail ..DPEEIPPEYLO  
hPAR3- 34-62 ..TLPIK / TERGAPPN SFEFFPFSALEGWTGA..  
hPAR1- 37-65 ..TLDPR / SPLLRNPNNDKYEPFWEDEEKNESG..  
hPAR2- 32-62 ..SSKGR / SLIGKVDGTSHTVTKGVTVETVFSVD..  
A

Cleavage site

FIGS. 5A & 5B

Surface staining of M1-epitope  
+/-  $\alpha$ -thrombin at 37°C for 5 minutes

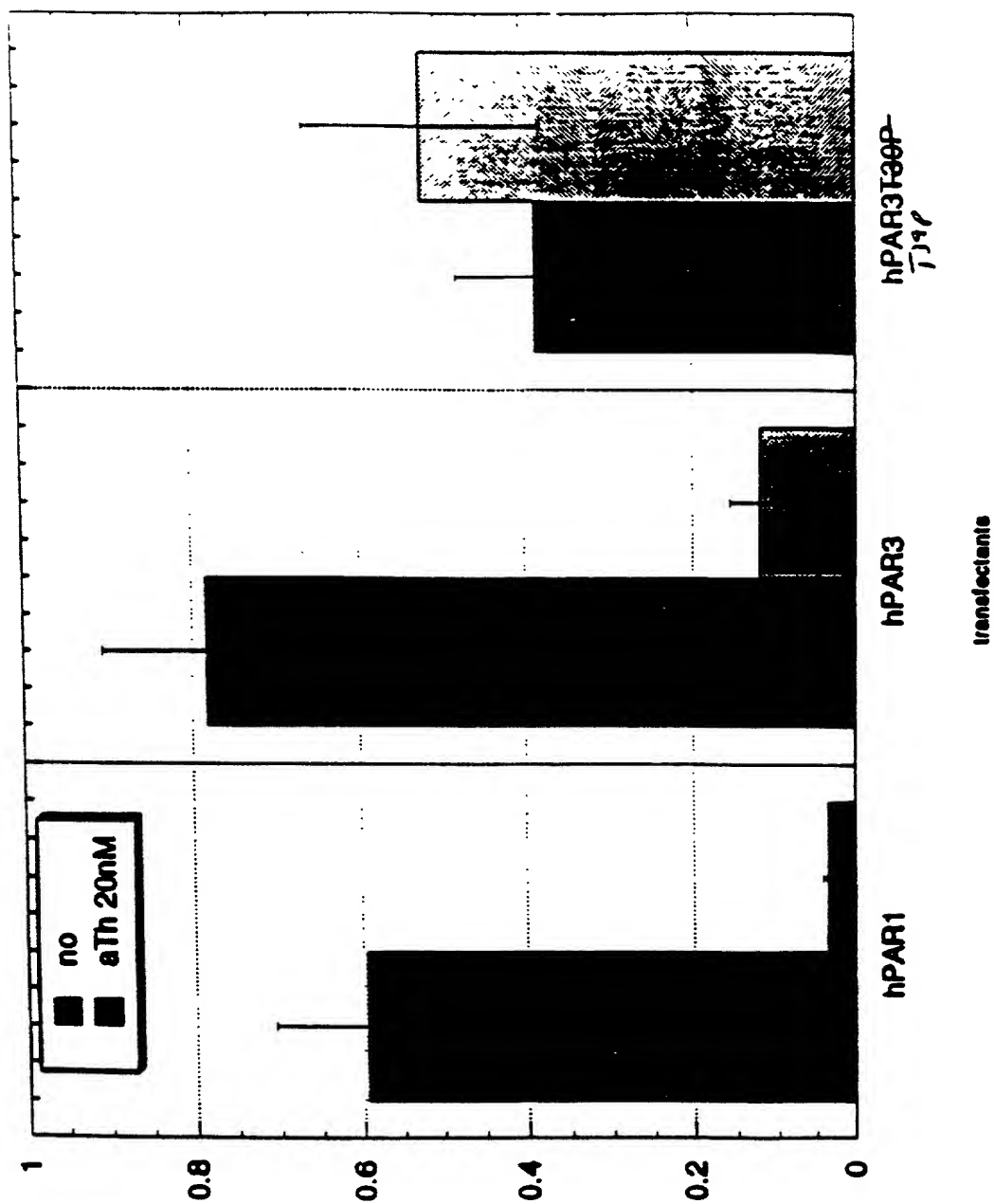


FIG. 6

# Signaling of PARs effects of Gα16

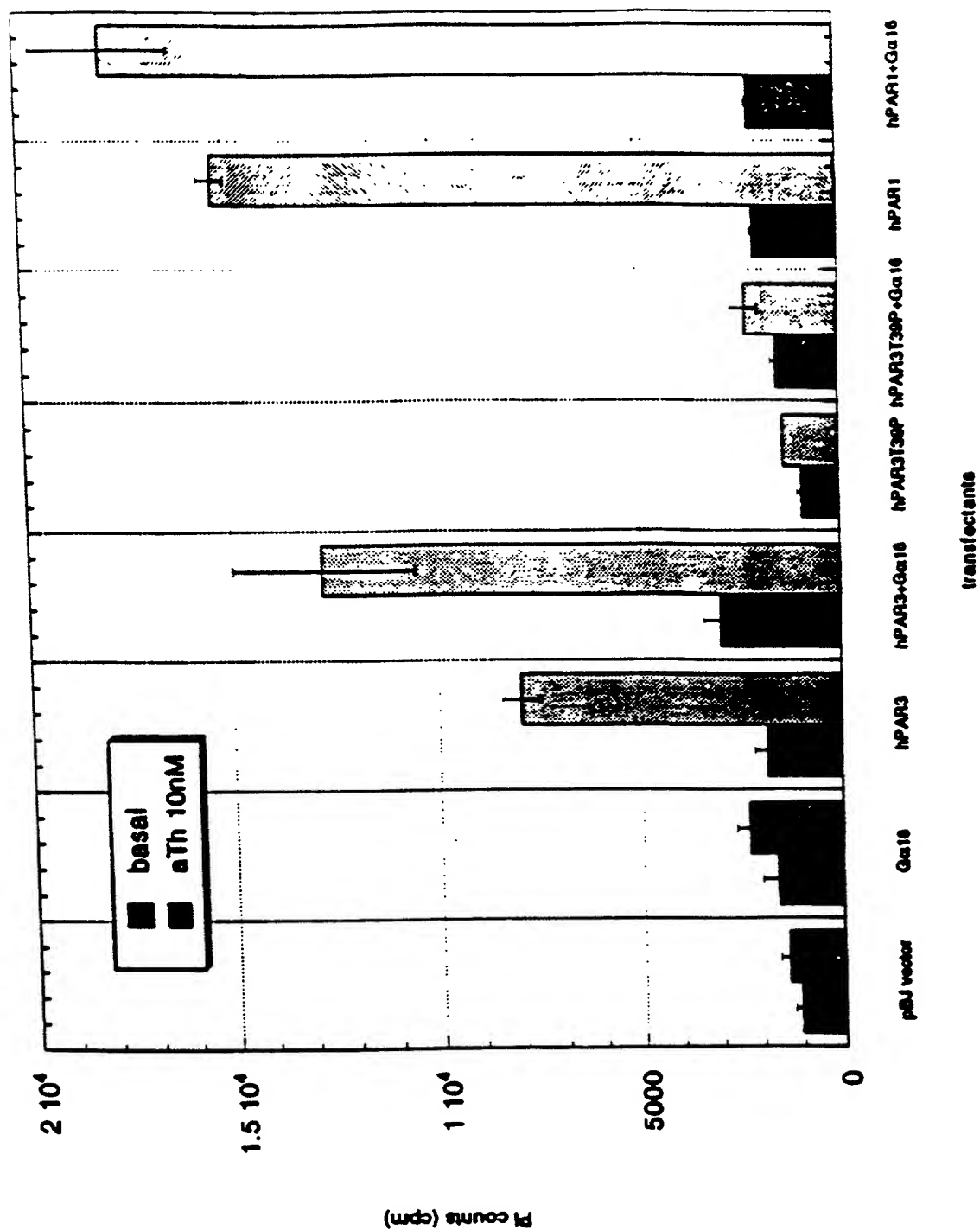


FIG. 7

$\alpha$ -thrombin dose response curve

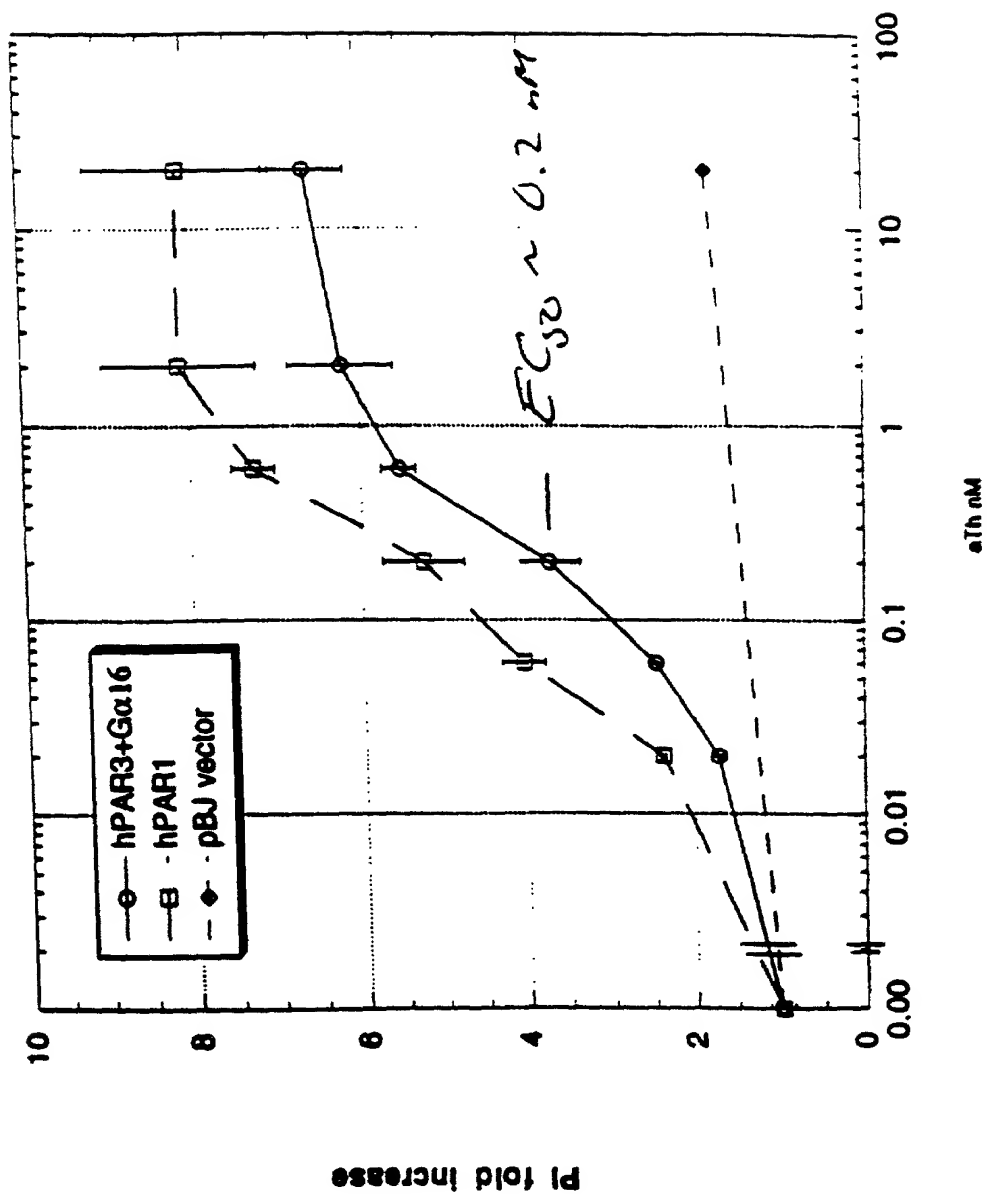


FIG. 8



$\gamma$ -thrombin dose response curve

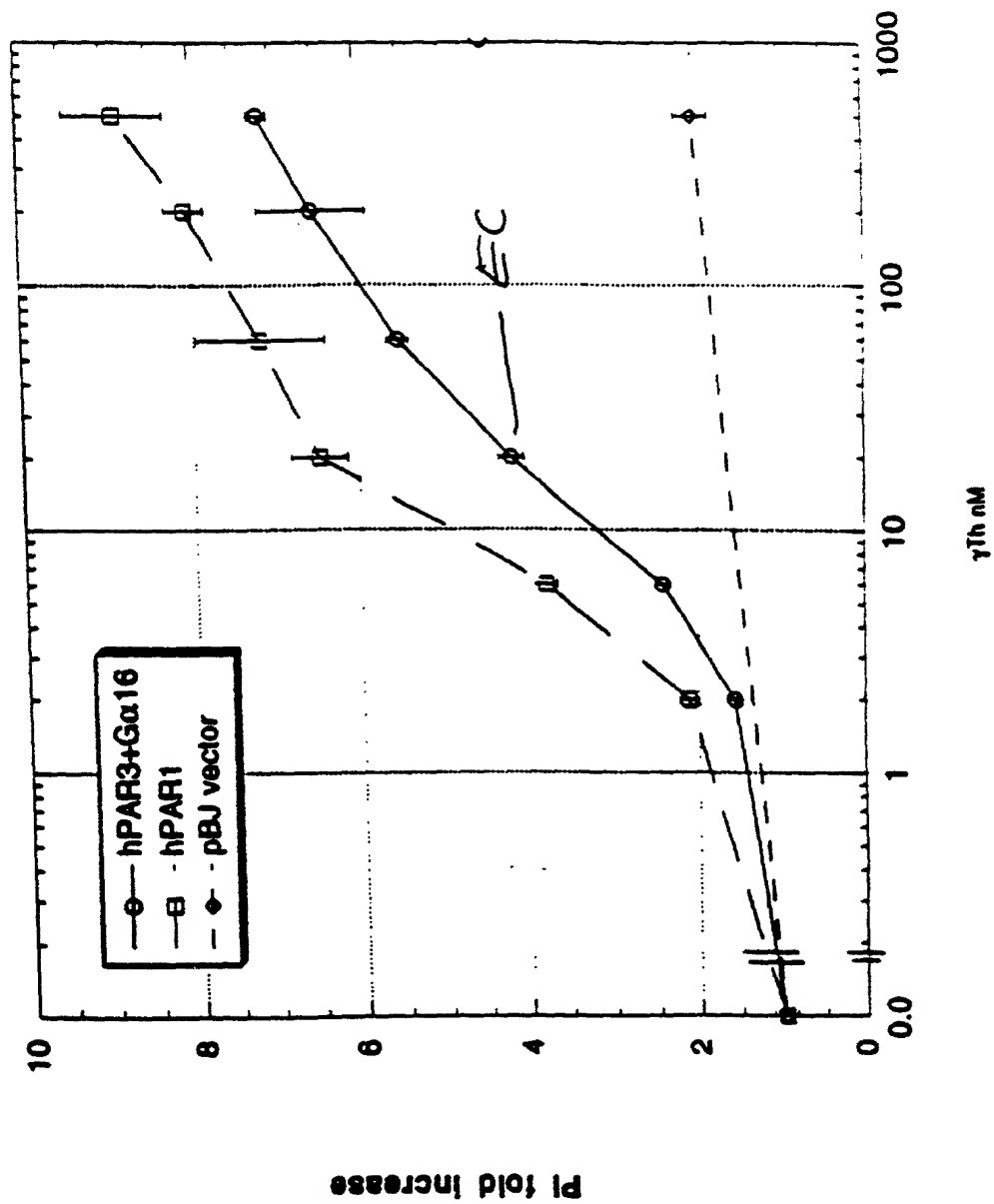


FIG. 9

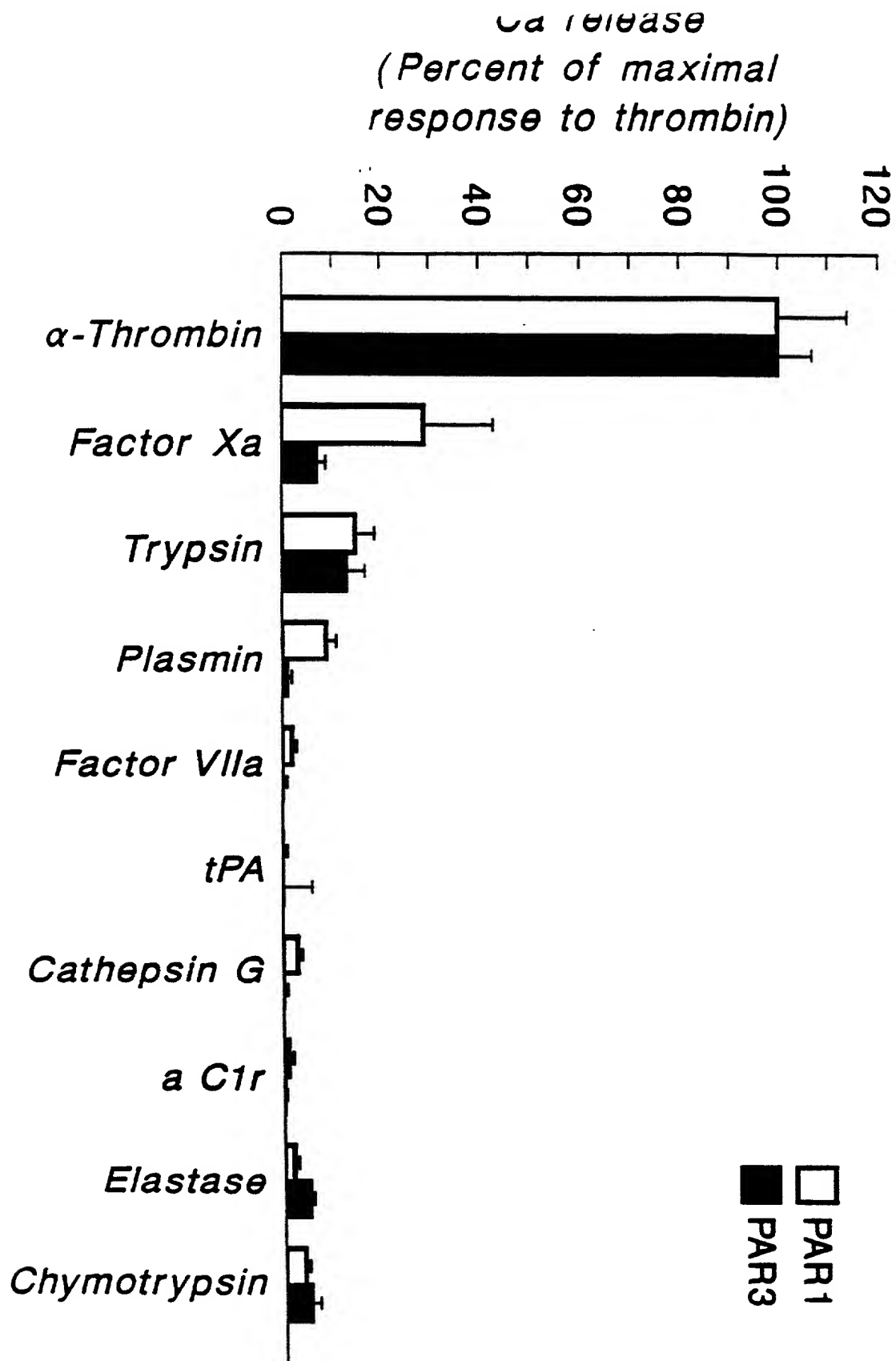


FIG. 10

## COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

## PROTEASE ACTIVATED RECEPTOR 3 AND USES THEREOF

the specification of which

☐ is attached hereto.

☒ was filed on October 30, 1996

☒ as Application Serial No. 08/742,440

☒ and was amended on \_\_\_\_\_.

☐ was described and claimed in PCT International Application No. \_\_\_\_\_  
filed on \_\_\_\_\_ and as amended under PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Karl Bozicevic, Reg. No. 28,807; Carol L. Francis, Reg. No. 36,513; Bret E. Field, Reg. No. 37,620; Pamela J. Sherwood, Reg. No. 36,677; Reginald J. Suyat, Reg. No. 28,172; Hans R. Troesch, Reg. No. 36,950; Grant D. Green, Reg. No. 31,259.

Address all telephone calls to Karl Bozicevic at telephone number 415/322-5070.

Address all correspondence to Karl Bozicevic, Fish & Richardson P.C., 2200 Sand Hill Road, Suite 100, Menlo Park, CA 94025.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: SHAUN R. COUGHLIN

Inventor's Signature: Shaun R. Coughlin Date: 2/14/97

Residence Address: 2 Turtle Rock Court, Tiburon, CA 94920

Citizen of: United States of America

Post Office Address: Same as Above

COMBINED DECLARATION AND POWER OF ATTORNEY CONTINUED

Full Name of Inventor: HIROAKI ISHIHARA

Inventor's Signature: *[Signature]* Date: 2/14/97

Residence Address: 1770 17th Avenue, San Francisco, CA 94122

Citizen of: Japan

Post Office Address: Same as Above

Full Name of Inventor: ANDREW CONNOLLY

Inventor's Signature: *Andrew Connolly* Date: 2/14/97

Residence Address: 1427 Schrader Street, San Francisco, CA 94117

Citizen of: United States of America

Post Office Address: Same as Above

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